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STUDIES ON THE IMMUNE RESPONSE OF THE OVINE RESPIRATORY TRACT TO PARAINFLUENZA 3 VIRUS

by

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Thesis presented for the Degree of Doctor of Philosophy of the University of Edinburgh in the Faculty of Medicine.

March, 1975.

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SUMMARY

Ovine IgG, IgM and IgA and antisera specific for these immunoglobulins were prepared. These reagents were used to estimate immunoglobulin levels in sheep sera and certain other body fluids including the respiratory secretions. The results showed that a secretory IgA system existed in the ovine respiratory tract. Furthermore IgA antibodies to Mycoplasma ovipneumonia were identified in the lung fluid of a sheep clinically affected with pulmonary adenomatosis.

IgA antibodies specific for Parainfluenza 3 were demonstrated in the respiratory secretions of lambs which had been experimentally infected with the virus.

A large molecular weight non-immunoglobulin substance, which inhibited Parainfluenza 3 and three other paramyxoviruses, was also identified in the respiratory secretions of both conventionally reared and specific pathogen free lambs. It is suggested that in certain reports non-specific inhibitors present in the nasal secretions of calves may have been confused with Parainfluenza 3 specific IgA antibody.

Attempts were made to define the protective role of nasal secretion antibody with vaccination-challenge experiments in specific pathogen free lambs. It was shown that live Parainfluenza 3 administered intranasally stimulated comparable serum antibody titres but higher nasal secretion titres than the same dose of live virus given intramuscularly. Inactivated virus inoculated without adjuvant by either route stimulated low or undetectable serum titres and no nasal antibody. Immunity to aerosol challenge, as assessed by viral shedding from the nose and changes in post challenge antibody titres, was best conferred by intranasal inoculation with live virus. Hence there was some evidence that the presence of antibody in

the nasal secretions reduced the susceptibility to infection.

In subsequent experiments it was found that intramuscular inoculation of inactivated Parainfluenza 3 in complete Freund's adjuvant stimulated high serum and nasal secretion titres, which protected against challenge. However the nasal secretion antibody was IgG_1, which was possibly selectively transferred from serum. This contrasted with the earlier finding of IgA antibodies in the respiratory secretions after intranasal inoculation of live virus and showed that a second mucous antibody system existed in the ovine respiratory tract.

Results from an experiment with young colostrum fed lambs indicated that maternal antibody alone could prevent infection with Parainfluenza 3 virus. This showed that the presence of cell-mediated immunity may not be essential to prevent sheep becoming infected with this virus. In this and a subsequent experiment it was demonstrated that colostral IgG passed into the nasal and lachrymal secretions of young lambs. This finding supported the earlier suggestion that IgG_1 is selectively transferred from serum into the nasal secretions of young lambs. It is suggested that the presence of maternal IgG_1 antibodies in the respiratory secretions of newborn suckled lambs could constitute an important defence mechanism against respiratory infections before local antibody synthesis begins at about two weeks of age.

SECTION I

INTRODUCTION

Sheep pneumonia was reported in 1959 as a world-wide disease of considerable economic importance (Shirlaw, 1959). More recent surveys have confirmed this view. Crempien et al, (1973) cite the American Sheep Producers Council who put the annual cost of ovine pneumonia in the USA at 37 million dollars. In India pneumonia was estimated to account for 15% of the mortality in one flock (Chattopadhyay et al, 1971), whereas lung lesions were observed in 30% of 113 sheep examined in another survey (Bhagwan and Singh, 1972). In Rumania 5.4% of 2708 sheep carcases had lung lesions, with a high incidence in young lambs (Ungureanu et al, 1970).

Recent estimates of the cost of sheep pneumonia in the United Kingdom are more difficult to obtain. During 1973 - 1974 0.4% of lungs obtained from 493,090 sheep slaughtered in the Glasgow and Edinburgh abattoirs were condemned (Sharp, personal communication). Obviously this figure greatly underestimates the total number of animals with lung lesions. A survey carried out between 1959 and 1961 showed that 10% of 715 deaths in sheep from 14 farms were caused by pneumonia (Hughes, 1964). Of probable equal or greater economic significance is the production loss due to subclinical disease and to non-fatal cases, although the cost of such loss is very difficult to assess. In view of the trend towards more intensive husbandry of sheep it is likely that the incidence of respiratory disease will increase.

The aetiology of acute respiratory disease in sheep is not fully understood. Various bacteria and mycoplasma have been incriminated, but at the time this work began Parainfluenza type 3 (PI_3) was the only virus which had been isolated from the respiratory tract of

sheep affected with acute respiratory disease (Hore, 1966; Ditchfield, 1966; St. George, 1969; Sharp, 1973). Recently, however, Belak and Palfi (1974) isolated Reovirus type 1 from lambs with pneumonia and adenoviruses, which possibly have a role in acute respiratory disease, have been recovered from sheep faeces (McFerran et al, 1969; McFerran et al, 1971; Sharp et al, 1974). Viruses have also been isolated from maedi and pulmonary adenomatosis two chronic respiratory diseases of sheep (Sigurdardottir and Thormar, 1964; MacKay, 1969 a & b) and in the case of maedi the disease has been experimentally reproduced with the virus (Gudnadottir and Palsson, 1967).

Although PI_3 had been associated with field outbreaks of respiratory disease and experimentally had produced pneumonia in lambs (Hore & Stevenson, 1967), at the time this study began little information was available on the immune response of sheep to the virus. This information was considered essential if a prophylactic vaccine was to be developed.

When this investigation started considerable interest was being paid to the role played by mucous antibody in preventing virus infections of the human respiratory tract. Indeed, protection against parainfluenza and rhinovirus infections in man had been reported to correlate more closely with nasal secretion antibody than with serum antibody (Smith et al, 1966; Perkins et al, 1969). However no information was available on the existence and possible function of antibody in ovine respiratory secretions. The main object of the work in this thesis was to determine whether antibody did exist in the respiratory secretions of the sheep. If so, the aim was to characterise this type of antibody and if possible to determine its role against PI_3 infection.

Review of the Literature

A complete review of the vast literature on the studies of local immune mechanisms in the respiratory tract will not be attempted.

Besides, recent comprehensive reviews covering aspects of this subject are available (Heremans, 1968; Tomasi and Bienenstock, 1968; The Secretory Immunologic System, 1969; Rossen et al, 1971; Airborne Transmission and Airborne Infection, 1973; Murray, 1973). Therefore only salient findings from the studies in man and laboratory animals most relevant to the work in this thesis will be described. However the literature available from studies in sheep and cattle will be reviewed in greater detail, but reports published later than 1970 will be discussed later with appropriate findings in this study. Emphasis will be placed on reviewing the studies of immunity in the respiratory tract, but where appropriate reports on the local immunity of other tracts may be quoted.

The concept of local immunity and local immunisation is by no means new. Following experiments before the First World War many thousands of people in the 1920s were successfully immunised with oral vaccines against cholera, typhoid and other bacterial dysentries (Besredka, 1930). Local immunity in the respiratory tract to Pneumoccocci infection was demonstrated in rabbits by Walsh and Cannon in the late 1930s (Walsh and Cannon, 1936; Walsh and Cannon, 1938). During the early 1940s reasonable evidence was presented for neutralising antibody to influenza virus in human nasal secretions (Francis, 1941; Francis and Brightman, 1941). Earlier reports exist for viral inhibitors in human nasal secretions but the inhibition was probably non-specific in nature (Amoss and Taylor, 1917; Burnet et al, 1939). After extensive studies on murine influenza in the early 1950s, Fazekas de St. Groth and Donnelly (1950) concluded that resistance to infection correlated with the antibody levels in bronchial washings and was independent of serum antibody. However despite these and other investigations, little

further interest was paid to the possible importance of local immune mechanisms in the respiratory tract until about ten years ago.

Interest in local immunity was re-awakened by the discovery of human IgA. Detected first in the serum (Heremans et al, 1959), IgA was shown to be the predominant immunoglobulin in most human secretions, where it was present as a larger, 11S molecule, attached to an additional antigenic component (Hanson, 1961; Tomasi and Zigelbaum, 1963; Tomasi et al, 1965). This extra protein, now termed the secretory component, is not present on 7S serum IgA but occurs free in the secretions of newborn children and agammaglobulinaemic patients (Tomasi and Bienenstock, 1968).

Since IgA is the predominant immunoglobulin in the secretions whereas IgG is the major serum immunoglobulin, it follows that IgA is either synthesised locally in the mucosa or else it is selectively transported from serum. A considerable body of evidence has been accumulated by a variety of techniques to show that IgA in the secretions is locally produced. Radiolabelled amino acids fed in vitro to organ cultures of various glands or portions of gut have resulted in the production of radiolabelled IgA in the medium (Hurlimann and Zuber, 1968; Lawton et al, 1970). Radiolabelled IgA injected intravenously into human volunteers has resulted in little or no excretion of IgA into saliva or nasal secretions, suggesting that secretory IgA is not derived from serum IgA (Tomasi et al, 1965; Butler et al, 1967; Strober et al, 1970). Immunofluorescent investigations employing antisera specific for IgA have revealed that the bulk of plasma cells in the submucosa of the respiratory and gastrointestinal tracts contain IgA (Crabbe et al, 1965; Brandtzaeg et al, 1967; Tourville et al, 1969). IgA antibody production on a mucous surface is mainly confined to that portion of the tract which

received the antigenic stimulus (Ogra and Karzon, 1969 a). This last finding is evidence for a local stimulation - local production concept.

It is known that human IgA exists as a 7S monomer in the serum whereas in most other species serum IgA is a 9S dimer (Heremans and Vaerman, 1971). An additional polypeptide termed the J chain is present on 11S IgA and 19S IgM but not on 7S IgA or IgG (Halpern and Koshland, 1970; Mestecky et al, 1971). This protein was originally thought to function as a 'junction chain', connecting single molecules of the 2 immunoglobulins to form the IgA dimer and the IgM polymer. However this function has recently been criticised by Eskland and Brandtzaeg (1974) who have suggested that J chain is essential for the combination of IgM and IgA with secretory component.

Following detailed immunofluorescent studies the following mechanism has recently been proposed for the selective secretion of IgA and IgM into human secretions (Brandtzaeg, 1974 a and b). IgA dimers and IgM polymers are produced by plasma cells in the submucosa close to the epithelium. Serous type epithelial cells manufacture free secretory component which is distributed in the cytoplasm and on the membranes of these cells. Only immunoglobulins with the J chain (i.e. IgA and IgM) attach to the secretory component on the epithelial cells and are secreted into the gland lumen. IgA predominates over IgM in the secretions because of the relative abundance of IgA producing plasma cells in the submucosa. In addition, according to Brandtzaeg (1974 b), IgM does not bind as avidly to secretory component as IgA.

Soon after the discovery of IgA, the anti-viral activity in human nasal secretions was associated with this type of immunoglobulin. (Artenstein et al, 1964; Bellanti et al, 1965). Since then numerous studies have been carried out in man comparing the antibody response

in the respiratory secretions and serum following vaccination by either the respiratory or parenteral routes. Thus antibody activity, usually shown to be due to IgA, has been demonstrated in the nasal secretions following natural infection or vaccination with many viruses including parainfluenza (Smith et al, 1966), influenza (Mann et al, 1968), rhino (Douglas et al, 1967) and polio (Ogra and Karzon, 1969 b).

In general it has been shown that both live and inactivated viral vaccines stimulate higher nasal secretion titres when administered by the respiratory route (Kasel et al, 1969; Waldman et al, 1968; Downie, 1973). Intranasal vaccination with live virus results in greater nasal secretion titres than inactivated virus administered by the same route (Downie, 1973). Furthermore it has been shown with rhinovirus and parainfluenza virus type 1 that protection correlates more closely with nasal antibody than with serum antibody (Cate et al, 1966; Perkins et al, 1969; Smith et al, 1966).

Locally produced IgG and IgA type antibody has been found in broncho-alveolar washings obtained from volunteers immunised with an aerosol influenza vaccine (Waldman et al, 1973). After natural infection or respiratory immunisation IgA antibody has also been demonstrated in sputum, saliva and tears (Mann et al, 1968; Douglas et al, 1967; Waldman et al, 1970 a). The local antibody response induced with inactivated viral vaccines is largely confined to that gland or mucous surface on which antigen is deposited. Thus intranasal (IN) vaccination with inactivated poliovirus stimulated antibody in the nasal secretions but not in the gut (Ogra and Karzon, 1969 b). Ogra and Karzon (1969 a) also immunised children who had double-barrelled colostomies with inactivated poliovirus. The response to local immunisation of the distal colon was limited to the distal segment. No antibody response was detected in the serum, nasopharynx and the non-immunised proximal segment of the

colon. Similar findings have been reported in the respiratory tract. Vaccination with inactivated influenza virus by nose-drops resulted in relatively high nasal antibody and low sputum titres, whereas vaccination by an aerosol of small droplets stimulated high sputum and low nasal secretion titres (Waldman et al, 1970 b).

It has been reported that IgA antibodies are "less specific" than IgG. Nasal secretion IgA antibody cross neutralised closely related strains of influenza virus, whereas serum IgG from the same individual did not (Waldman et al, 1970 a). However secretory IgA may function as a non-specific inhibitor because of its high sialic acid content (Alford et al, 1967). Buscho et al (1972) reported that IgA did not cross neutralise closely related strains of rhinovirus and suggested the effect seen with influenza might reflect an unknown antigenic relationship of the virus strains tested.

In man there appears to be little immunological memory in the secretory IgA system (Smith et al, 1967; Ogra and Karzon, 1969 a; Buscho et al, 1972). However studies in calves (Marshall and Frank, 1971), monkeys (Mattson et al, 1974) and mice (Gerbrandy and van Dura, 1972) do show an anamnestic antibody response in the respiratory secretions.

More recently cell mediated immunity has been reported in the respiratory tract. By means of the macrophage migration inhibition test, sensitised lymphocytes have been detected in lung washings obtained from guinea pigs and men, immunised with a variety of antigens including influenza virus (Henney and Waldman, 1970; Waldman et al, 1972; Jurgensen et al, 1973; Spencer et al, 1974). In these studies it was found that alveolar washing lymphocytes were sensitised to a greater extent if the antigen was administered by the respiratory route. However splenic or circulating lymphocytes were more readily

stimulated by parenteral immunisation. This suggests that a local cell mediated response as well as a local antibody response follows respiratory immunisation. An anamnestic alveolar and splenic cell mediated response has recently been found in guinea pigs immunised either IN or by footpad with influenza vaccine (Gadol et al, 1974). The secondary response occurred in both alveolar and splenic lymphocytes regardless of the route of the second dose of vaccine. However both the primary and secondary response in the alveolar lymphocytes was greater in the animals immunised by nose-drops. However the role of cell mediated immune responses in the respiratory tract to virus infections remains to be defined.

In ruminants secretion antibodies were first extensively studied in the bovine reproductive tract with regard to vibriosis, trichomoniasis and brucellosis (Pierce, 1959). Antibody to these organisms could be detected in the vaginal mucus often in the absence of serum antibody (Florent, 1947; Pierce, 1946; Pierce, 1947). Thus it soon became apparent that a mucous antibody system which was effective in vivo existed in the reproductive tract of the cow (Pierce, 1950).

Although the first reports of attempts to measure antibody in bovine nasal secretions do not appear until 1970 (Morein, 1970; Gates et al, 1970; Goedmans, 1970; Duncan and Thompson, 1970; Provost, 1970), earlier investigations had shown the efficacy of vaccinating calves IN with attenuated strains of PI_3 virus (Bogel and Liebelt, 1963 a). Bogel and Liebelt (1963 b) found that IN vaccination produced a serum haemagglutination inhibition (HI) antibody response equal to that stimulated by simultaneous IN and IM inoculations. However no serum HI increase was observed following IN vaccination, if the calves had very high maternal antibody titres and revaccination was essential

6 months later.

Gates et al (1970) and Gutekunst et al (1969) found that calves which had been vaccinated IN or naturally exposed to PI_3, shed little virus after experimental or natural challenge. However IM vaccinated calves shed large amounts of virus after challenge and in one experiment showed clinical signs of respiratory disease (Gutekunst et al, 1969). It seemed therefore that the vaccine was more effective when given IN and neither group of workers could correlate serum antibody levels with protection. Gates et al (1970) attempted to measure antibody in nasal washings but found anti-viral activity to PI_1 as well as PI_3 and concluded that the effect was not due to antibody.

Duncan and Thomson (1970) found antibodies to Pasturella haemolytica in the nasal washings and sera of calves. Nasal washing antibodies were detected consistently in calves exposed to aerosols of P. haemolytica whereas nasal antibody was not found in parenterally immunised animals.

About this time the presence of IgA was firmly established in the bovine (Vaerman et al,1969; Mach et al, 1969; Porter and Noakes, 1970). In the secretions IgA was soon shown to be attached to secretory component and to be the predominant immunoglobulin in most of these fluids including the respiratory secretions (Morein, 1970; Mach and Pahud, 1971; Watson and Lascelles, 1971). Fluorescent and in vitro synthesis studies have further supported the concept of an IgA mediated bovine secretory immune system, similar to that described in man and other species (Mach and Pahud, 1971; Yurchak et al,1971; Butler et al, 1972). However, except for one account describing PI_3 specific IgA antibodies in the nasal secretions of calves (Morein, 1970), no reports were available before 1971 on the role of IgA in

infectious diseases of the bovine.

Over the past decade considerable evidence has been presented supporting the concept of an IgA mediated local immune system in the sheep. Originally IgA was vaguely identified in sheep serum as a precipitin arc on immunoelectrophoresis corresponding with the position of IgA in human serum (Silverstein et al, 1963; Aaland et al, 1965, Pan et al, 1968). However Vaerman et al (1969), using a cross reacting, monospecific anti-human IgA serum, detected IgA in sheep serum. Since then IgA has been isolated from sheep serum, colostrum and saliva and monospecific antisera have been prepared for this immunoglobulin (Pahud and Mach, 1970; Curtain and Anderson, 1971; Lascelles and McDowell, 1970). In common with other species this immunoglobulin has been shown to be attached to a secretory component in the secretions (Pahud and Mach, 1970), whereas in serum it exists largely as a 9S dimer as in the bovine and goat (Mach and Pahud, 1971; Pahud and Mach, 1970). IgA exists at low concentrations in sheep serum but is present in many sheep body fluids (Watson and Lascelles, 1971; Pahud and Mach, 1970). However before 1971, saliva was the only body fluid examined in which IgA was shown to be the predominant immunoglobulin (Pahud and Mach, 1970). Fluorescent studies have shown that the plasma cells found near the glandular epithelium of the mammary gland (antigenically stimulated) or of the intestine are mainly of the IgA type (Lee and Lascelles, 1970; Curtain and Anderson, 1971).

Evidence for local antibody production in the mammary gland of the ewe has been reported by Lascelles and his collaborators. Comparing normal glands and glands which had been infused with bacterial antigens, these investigators found higher antibody titres in the whey milked from the infused halves of each udder (Lascelles et al, 1966). In addition the whey antibody titre was greater than the serum titre.

Later the local antibody was shown to be distinct from IgG and IgM and was eventually identified as IgA (Outteridge et al, 1968 a; McDowell and Lascelles, 1969; Lascelles and McDowell, 1970).

Antibody to helminth Oesophagostomum columbianum has been reported in intestinal mucus extracted from sheep infested with this parasite. Antibody titres were higher in the mucus than the serum. Moreover the highest mucous titres were present in those sections of gut which contained most parasites, strongly suggesting that the mucous antibody was of local origin (Dobson, 1966).

However despite these investigations there are few reports as to the possible function of the local immune system in the sheep. In one pertinent investigation Outteridge et al (1968 b) infused one side of 16 ewes' udders with a staphlococcal cell toxoid vaccine. Six weeks later both glands were challenged with virulent organisms. Twelve non-infused glands showed clinical signs of mastitis, whereas the infused sides remained clinically normal.

SECTION II

The first part of this section describes the techniques employed in isolating sheep IgG, IgM and IgA and the preparation of antisera specific for these immunoglobulin classes. In part 2 the methods used for sampling various sheep body fluids are described and, using the specific antisera prepared in part 1, results of the concentrations of immunoglobulins estimated in each of these fluids are presented.

PART I

Preparation of Immunoglobulins and Specific Antisera

Introduction

Sheep IgG, IgM and IgA and antisera specific for these immunoglobulins were prepared using the techniques of salt precipitation and ion exchange and gel filtration chromatography. These are commonly used methods for the preparation of purified immunoglobulins and have been employed by others who have prepared ovine immunoglobulins (Pahud and Mach, 1970; Hudson et al, 1970; Reid et al, 1971).

Materials and Methods

Serum

About 4 l. of blood were collected from the neck vessels of 3 sheep at slaughter. Pre-suckling blood samples were obtained from the jugular vein of several newborn lambs. Blood samples were allowed to clot and were left overnight at room temperature before the serum was harvested. Serum was stored at -20°C.

Lung Fluid

Lung fluid was obtained from clinical cases of pulmonary adenomatosis by elevating the affected sheep posteriorly and collecting the fluid flowing from the nostrils. This fluid was filtered (Whatman no. 1) and centrifuged (1000g, 10 minutes)to remove gross debris before being stored at -20°C.

Colostrum

This was collected by milking several ewes within 36 hours of lambing. Colostrum was either stored at -20°C until used or the whey was extracted within a few hours of milking.

Colostral Whey

Fat was first separated from the whole colostrum by centrifugation (1000g, 20 minutes). The lower aqueous layer (about 70% of the total volume) was removed, warmed to 37°C and 2.5 ml of rennin was added to each 100 ml. After about 2 hours incubation, the resultant clot was broken up and following further centrifugation (1000g, 20 minutes), the supernate was removed and stored at -20°C as whey.

Preparation of gamma globulins

Gamma globulins were precipitated from whey or lung fluid by the slow dropwise addition of saturated ammonium sulphate (SAS) to a stirred volume of the fluid until the final mixture contained 40% saturated SAS. The resulting cloudy solution was left to flocculate for about one hour before centrifugation (1,500g, 10 minutes). The precipitate was washed twice with 40% saturated SAS and redissolved in a minimal volume of distilled water.

Ion Exchange Chromatography

This was carried out using either diethylaminoethyl (DEAE) cellulose or DEAE Sephadex A 50. Acid washed cellulose was equilibrated in 0.01M Na phosphate (PO_4) buffer pH 7.6 and packed in columns. Columns were eluted stepwise at 4°C using 0.01M PO_4 pH 7.6 as the starting buffer and the following Na Cl concentrations in 0.01M PO_4 at pH 7.6 :- 1) 0.01M 2) 0.03M 3) 0.05M 4) 0.07M 5) 0.1M and 6) 0.2M. The sample applied to each column was equilibrated against the starting buffer by dialysis or pressure dialysis. Individual column

dimensions and flow rates are specified in the text. Details of the methods used in preparing these buffers are described in Appendix 1.

DEAE Sephadex chromatography was carried out at room temperature as described by Reid et al (1971) with the following modifications. The Sephadex was equilibrated with 0.075M PO_4, pH8 and subsequent elution steps were performed using 0.2M PO_4 and 0.2M PO_4, 0.2M Na Cl buffers both at pH8.

Gel Filtration

This was carried out in columns with Sephadex G200 or Biogel A 1.5M.

Appropriate weights of Sephadex were swollen in buffer (0.1M Tris. 1M Na Cl pH8 with 0.02% sodium azide), degassed and packed into columns, the parameters of which are given at appropriate points in the text.

Five hundred ml of Biogel were washed with several litres of phosphate buffered saline pH 7.2 (PBS) and an appropriate volume was packed in a 2.5 cm diameter glass column forming a bed length of 70 cm. This column was eluted at 4°C with an upward flow of PBS at 12 ml per hour.

All column effluents were monitored at 254nm with an LKB Uvicord and fractions were collected by means of a fraction collector.

Preparation of Antisera

One ml of sheep serum or of an immunoglobulin preparation (1-2 mg/ml) was injected intramuscularily (IM) with 1.0 ml of Freunds complete adjuvant (FCA - 20% Falba in Bayol F with 10 µg/ml freeze dried mycobacteria) into New Zealand White rabbits. Three or 4 inoculations were administered at 14-day intervals and thereafter 'booster' injections were given as necessary. The sera from several bleeds from different rabbits were pooled and stored in aliquots at -20°C.

Agar gel diffusion

This was carried out in a similar manner to Ouchterlony (1953). Immunodiffusion was done with a variety of well patterns in 1% Ionagar No. 2 in PBS, which had been layered in 10 ml aliquots onto glass plates (8 x 8 cm). The plates were held in humid boxes at room temperature until the precipitin lines had formed. Slides that were preserved were soaked overnight in PBS, washed with distilled water and dried under damp filter paper at 37°C overnight. The dried agar slides were fixed for 5 minutes in 2% aqueous acetic acid, stained with 0.5% amido black in methanol/glacial acetic acid 9 : 1 for 7 minutes and decolourised with methanol/glacial acetic acid 9 : 1 for 15 minutes.

Immunoelectrophoresis (IEP)

A micromethod similar to that described by Scheideggar (1955) was employed. Barbitone acetate buffer (0.05M Na Barbitone, 0.08M Na Acetate pH 8.6) was used in the tank. Ten ml of 1% Ionagar No. 2 in $\frac{1}{4}$ strength buffer was layered onto glass plates (8 x 8 cm). After the agar had hardened, the samples were applied to small wells cut in the agar and a potential difference of 100 volts was applied across the slides for 1 hour. Troughs were then cut and the antisera applied. Some slides were preserved as previously described.

Protein Determinations

These were kindly done by Mr. Dawson (Chemistry Department) by a micro-Kjeldahl method, using the factor 6.25 for converting nitrogen to protein.

Experimental Procedure

The procedure used in preparing each immunoglobulin and its monospecific antiserum was as follows: first, rabbit antisera to pooled

sheep serum were raised and used to detect the presence of IgG and IgM by IEP analysis. Secondly, using colostral whey as the starting material, an attempt was made to prepare crude IgM, IgG and IgA and antisera to these fractions. Monospecific antisera to IgG and IgM were produced by this method but no activity to IgA was detected. Sheep IgA was detected with a monospecific, cross-reacting anti-bovine IgA serum and so a monospecific serum to sheep IgA was eventually prepared. Thirdly, these monospecific antisera were used in immunodiffusion tests to analyse the immunoglobulins present in fractions from the chromatography columns. Thus the standard preparations of IgG, IgM and IgA were obtained from colostrum, serum and lung fluid respectively. In order to simplify presentation, the preparation of each immunoglobulin and its specific antiserum is described separately.

Results

Preparation of anti-IgG and IgG

Thirty ml of sheep colostral whey globulin were dialysed for 72 hours at $4^{o}C$ against several litres of 0.01M PO_4 pH 7.6. This dialysis resulted in a slight precipitate which was removed by centrifugation (2,000g, 15 minutes) before the solution was applied to the DEAE cellulose. Stepwise elution of the column as described in 'materials and methods' resulted in a series of protein peaks exemplified in Fig I. IEP analysis of the first 4 peaks with anti-whole sheep serum revealed a single precipitin arc in the IgG_1 position (Fig 2). Fractions composing the first and second peaks were pooled and 1 ml aliquots were inoculated into rabbits. The resulting antisera reacted with sheep serum or whey producing a single line on gel diffusion and, on IEP with serum, produced the characteristic lines with IgG_1 and IgG_2 (Fig. 3A).

Two ml of the IgG preparation were filtered through Sephadex G200

Fig. I Fractionation of colostral whey globulin on DEAE cellulose.

Sample: 53 ml 43.9 mg protein/ml. Flow rate: 60 ml/hour.

Column: 2.5 x 25 cm. Fraction size: 10 ml.

Buffers: 1, 0.01 M NaCl; 2, 0.03 M NaCl; 3, 0.05 M NaCl; 4, 0.07 M NaCl; 5, 0.1 M Na Cl. All in 0.01 M PO_4, pH 7.6.

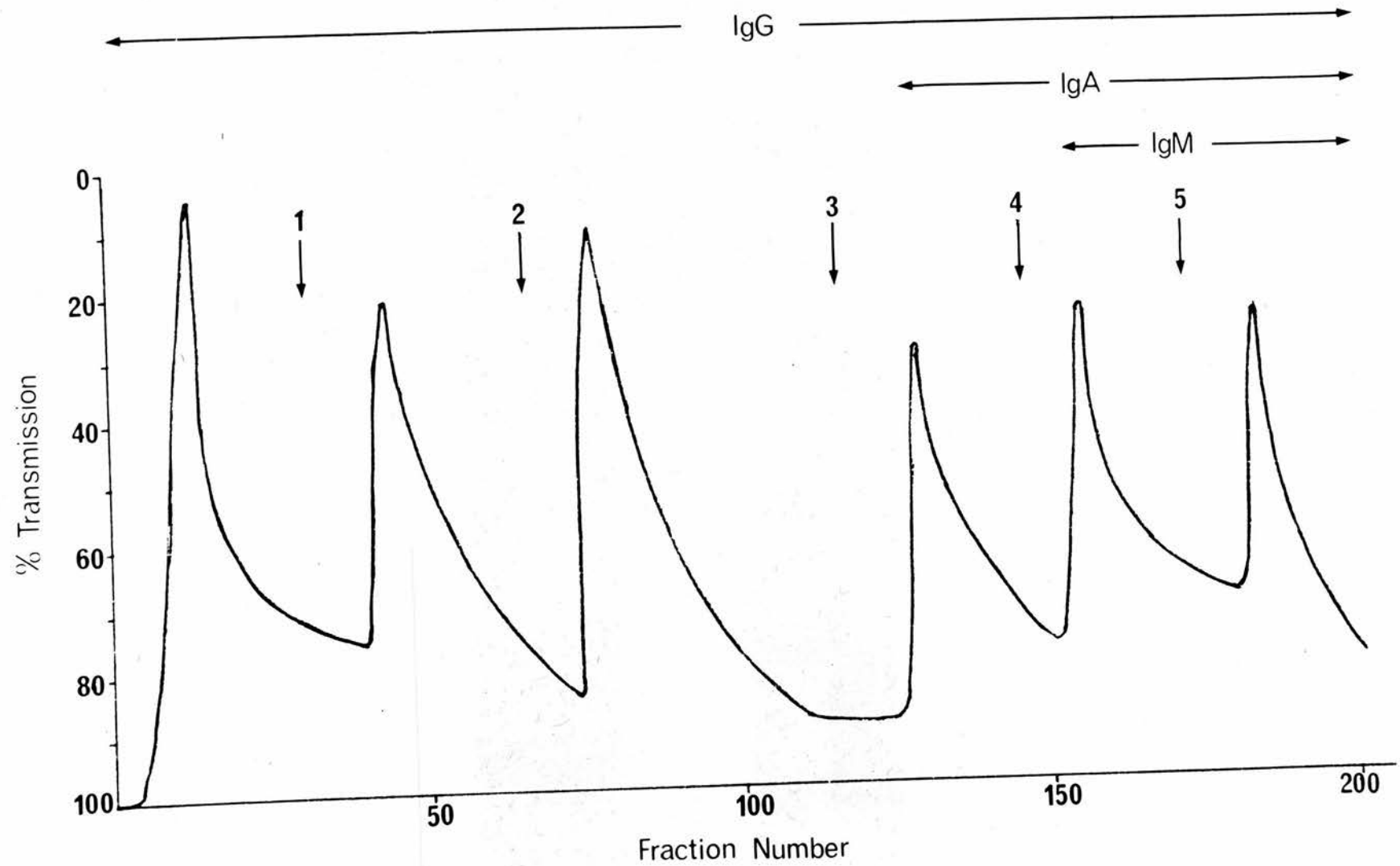

IgG
IgA
IgM
1
2
3
4
5
0
20
40
60
80
100
% Transmission
50
100
150
200
Fraction Number

Fig. 2 Immunoelectrophoresis of IgG preparation.

Upper and lower wells; IgG preparation.

Centre well; sheep serum.

Troughs; anti-whole sheep serum.

The anode is on the left.

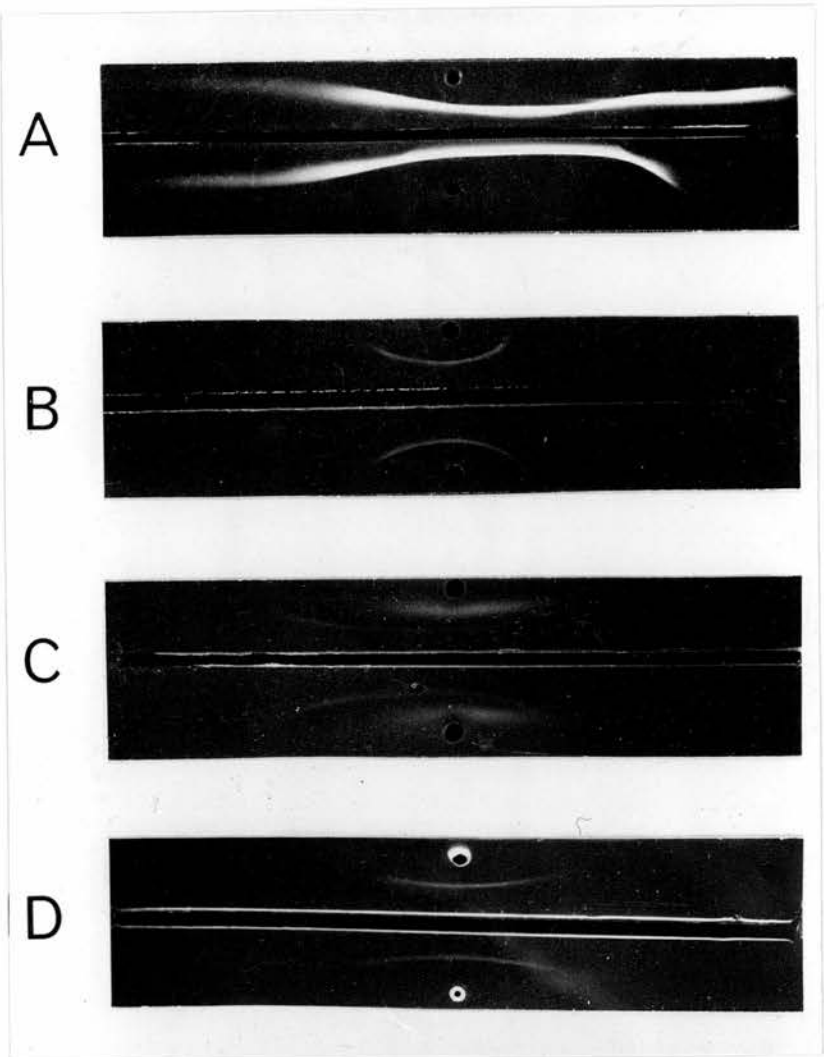


Fig. 3 Immunoelectrophoretic analysis of specific antisera.

A upper well, sheep serum; lower well, sheep whey; trough, anti-IgG

B upper well, sheep serum; lower well, sheep whey; trough, anti-IgM

C wells, sheep whey; trough anti-IgA

D upper well, sheep serum (rich in IgA); lower well sheep whey; trough, anti-IgA diluted 1:4.

The anode is on the left.

(Column 90 x 2.5 cm, Flow 25 ml/hour) resulting in a single symmetrical protein peak. This preparation was used as the IgG standard for the single radial diffusion assay (Section II part 2).

Preparation of anti-IgM and IgM.

Anti-IgM Colostral whey globulin was filtered through Sephadex G200 (Column 70 x 2.5 cm, Flow 14 ml/hour) in 2 ml aliquots. Crude IgM was obtained from the fractions contained in the leading edge of the exclusion peak. The relevant fractions from several elutions were pooled, concentrated in an ultrafiltration cell (Amicon filter XM 100), and injected in 1 ml aliquots into 2 rabbits. When reacted immuno-electrophoretically against sheep serum or whey, the antisera from these animals produced several precipitin lines including dense arcs in the characteristic IgG and IgM positions. A pool of antiserum was made specific for IgM, as judged by gel diffusion and IEP analysis, after absorption with IgG and precolostral lamb serum (Fig. 3B). The anti-serum was first absorbed with 0.4 ml of precolostral serum per ml and then anti-IgG activity was removed either by adding 0.28 mg IgG per ml or by passing 2 ml aliquots of the serum through a column of IgG covalently bound to sepharose beads (see below). Absorption reactions in solution were left to react overnight at 4°C or for 2 hours at 37°C, before the precipitate was removed by centrifugation (2,000g, 10 minutes).

IgM The euglobulins were precipitated from a 1.2 l pool of sheep serum by dialysis against running tap water for 3 days at 4°C. The precipitate was dissolved in 18 ml 0.1M Tris - NaCl buffer, pH 8, and filtered in 3 ml aliquots through Sephadex G200, (Column 90 x 2.5 cm, Flow 25 ml/hour) at room temperature. A single protein peak with a small trailing shoulder was produced by each sample at the exclusion limit of the column (Fig. 4A). Fractions forming the ascending

Fig. 4 Preparation of IgM.

A Filtration of serum euglobulins through Sephadex G200.

Column: 90 x 2.5 cm. Flow rate: 25 ml/hour.

Sample: 3 ml. ⊢⊣ = Fractions collected.

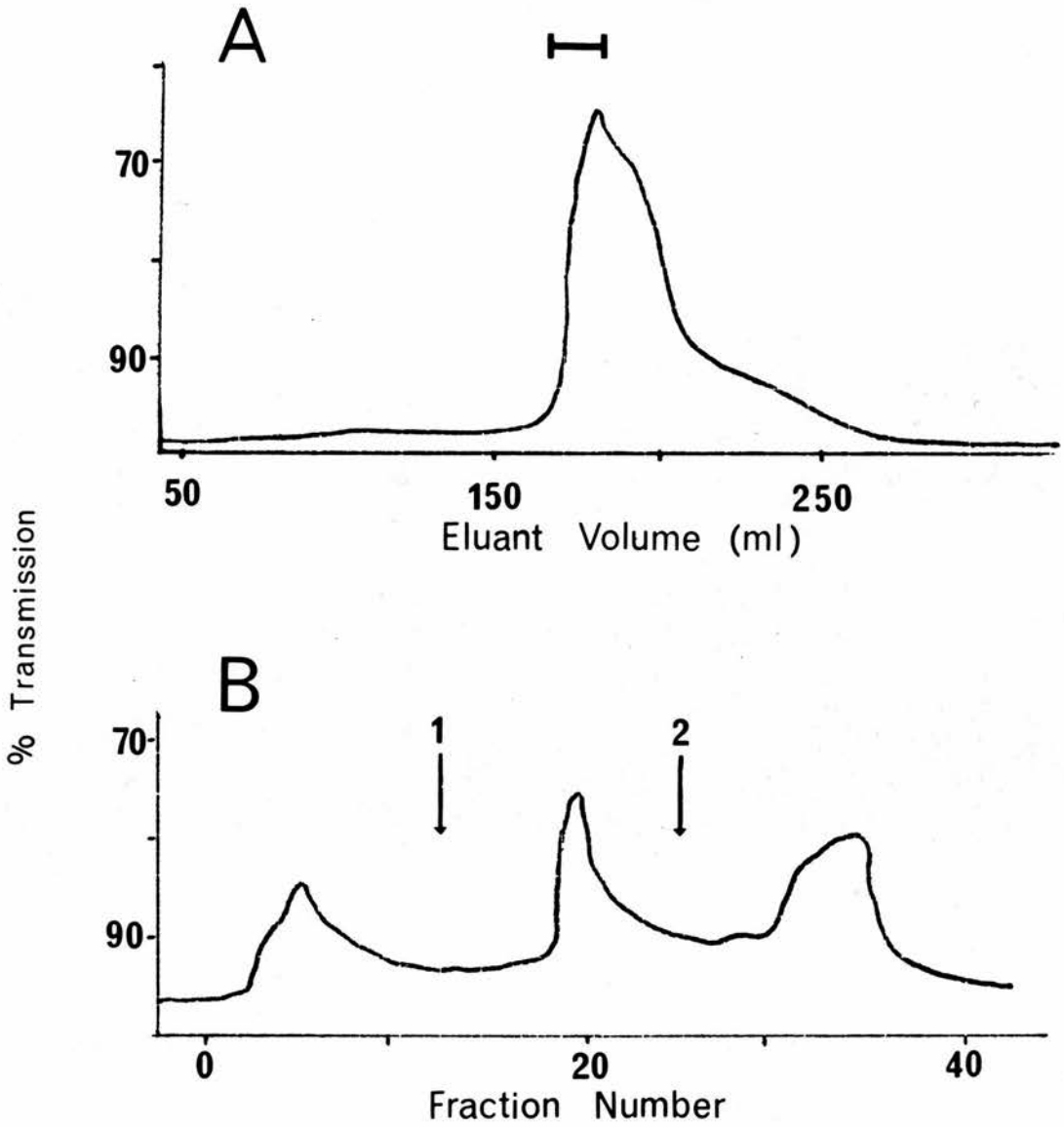
B Elution from DEAE Sephadex of IgM rich fractions pooled and concentrated from 6 filtrations similar to A.

Column: 20 x 1.5 cm. Flow rate: 20 ml/hour.

Fraction size: 4.5 cm. Sample volume: 20 ml.

Starting buffer: 0.125 M PO_4 pH8. Elution buffers:

1, 0.2 M PO_4 pH8; 2, 0.2 M PO_4, 0.2 M NaCl pH8.


A
70
90
50
150
250
Eluant Volume (ml)
% Transmission
B
1
2
70
90
0
20
40
Fraction Number

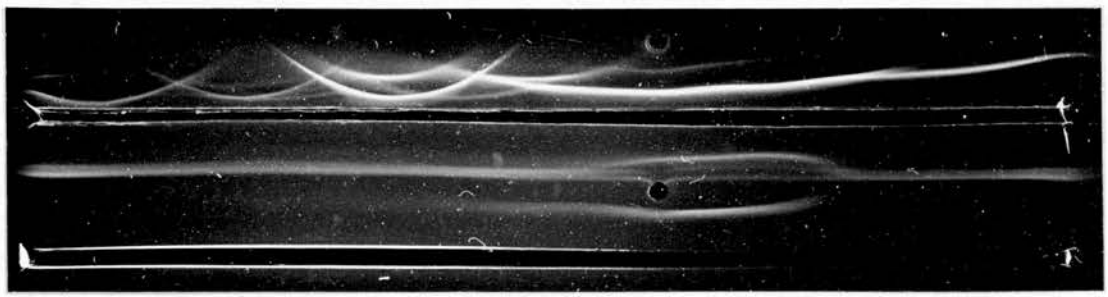


Fig. 5 Immunoelectrophoresis of IgM preparation.

upper well; sheep serum.

lower well; IgM preparation.

upper trough; anti-whole sheep serum.

lower trough; anti-IgM serum.

(The anti-IgM serum was absorbed in solution with a slight excess of IgG - hence the reaction between the troughs).

The anode is on the left.

portions of the first peak were pooled, concentrated by pressure dialysis against 0.125M PO_4, pH 8, and applied to a column of DEAE Sephadex (20 x 1.5 cm; Flow 20 ml/hour) equilibrated against the same buffer. After concentration the protein peaks eluted by the second and third buffers each gave a single line corresponding to IgM on IEP analysis with anti-sheep serum (Figs. 4B and 5). However on immunodiffusion a second faint precipitin line was observed. This may have been due to slight contamination with α 2 macroglobulin, a protein which has similar physicochemical properties to IgM, since later analysis with specific anti-IgG and anti-IgA sera did not reveal any precipitin lines.

The partially purified IgM preparation eluted by the second buffer was used as the IgM standard for single radial immunodiffusion and contained 3.02 mg of protein per ml.

Preparation of anti-IgA and IgA

Aliquots of colostral whey were filtered through Sephadex G200 as previously described. An attempt was made to raise crude anti-IgA serum by immunising rabbits with fractions eluted just after the first protein peak. These fractions had been reported to contain IgA (Pahud and Mach, 1970). Two rabbits were inoculated and bled over a 4 month period However IEP analysis of their sera with whey revealed no activity which could be ascribed to anti-IgA, although strong IgM and IgG precipitin arcs were observed.

A pig antiserum, monospecific for bovine IgA and which cross-reacted with sheep IgA was kindly supplied by Mr. T. Newby (Bristol University Veterinary School). This antiserum, together with the specific anti-IgG and anti-IgM sera, was used to monitor the distribution of immunoglobulins when colostral whey globulin was fractionated on DEAE cellulose, so that an IgA rich fraction could be prepared. Preliminary experiments carried out on small columns

(20 x 1.5 cms, Flow 30 ml/hour) with the buffer steps already described showed that, while IgG was present in all peaks, IgA was not desorbed until buffer 3 was applied while IgM was first eluted by buffer 4 (Fig. I). This experiment was repeated several times on a larger scale (columns 20 x 2.5 cm; Flow 60 ml/hour). Fractions eluted by buffers 3 and 4 were pooled, concentrated by pressure dialysis in PBS and filtered in 2 to 4 ml aliquots through a column of Biogel A 1.5M (Fig. 6A). IgA was present in the shoulder before the main peak, which contained the bulk of the contaminating IgG. IgA rich fractions were pooled and after concentration filtered through the same column, resulting in a single symmetrical peak, the first half of which was pooled, concentrated and injected into rabbits (Fig. 6B).

Antisera from these animals reacted with sheep whey on IEP analysis, producing 2 main precipitin arcs in the IgG and IgA positions. A fainter, faster migrating protein was also observed which appeared to fuse at its cathodic extremity with the IgA arc (Fig. 3C). This line was not detected when the antiserum was diluted 1:4 in PBS, and it was never detected with sheep serum (Fig. 3D). It was concluded that the arc was probably the result of anti-secretory component activity reacting with free secretory component in sheep whey. Specific anti-secretory IgA serum was obtained after absorption with IgG and pre-suckling lamb serum immunoadsorbents. On immunodiffusion with sheep and bovine whey, this serum and the anti-bovine IgA serum produced identical precipitin lines (Fig. 7).

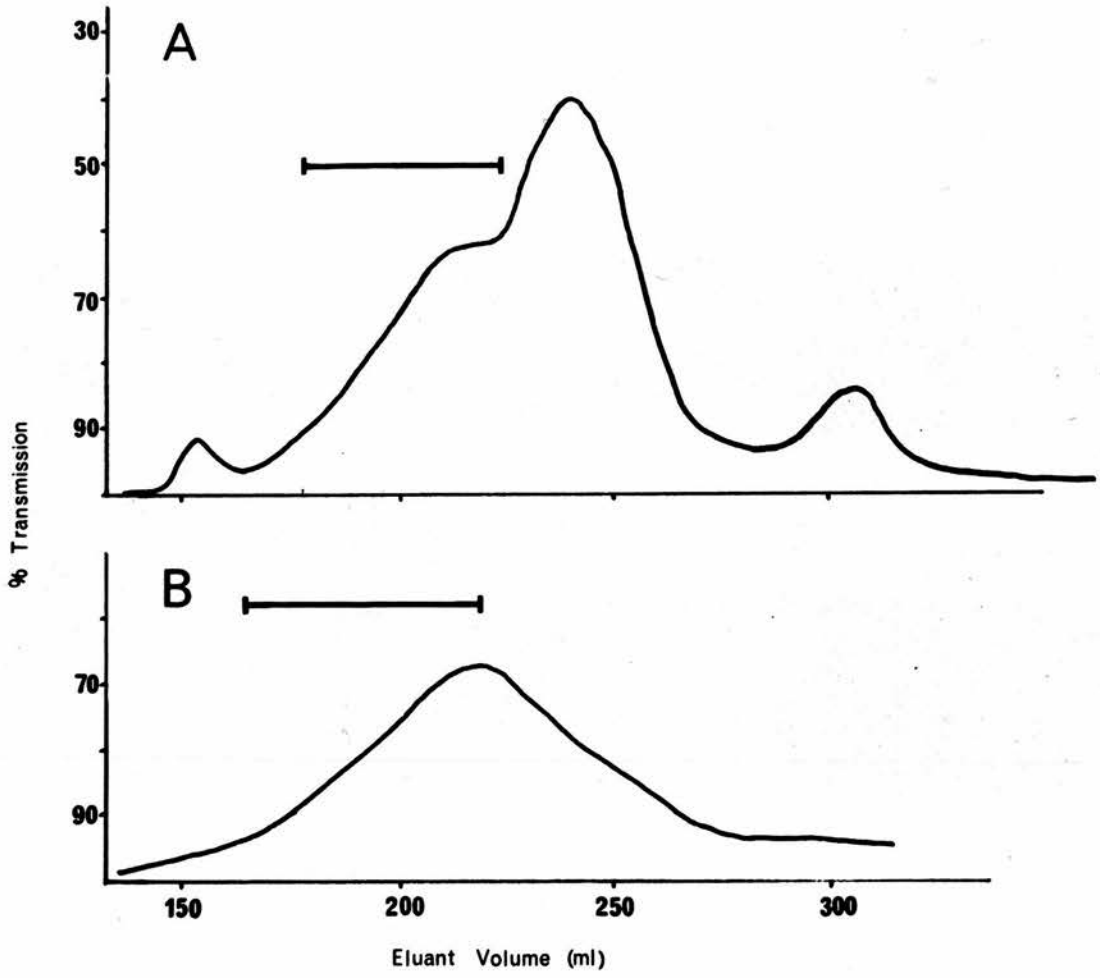
Despite the chromatography procedures described, some 50% of the protein in the IgA fraction prepared from whey was IgG. Later analysis of some other ovine body fluids (Section II, part 2) revealed that lung fluid from sheep with pulmonary adenomatosis contained no detectable IgM and had a high IgA:IgG ratio. Since this fluid could also be

Fig. 6 Partial separation of IgA by recycling through Biogel A 1.5 M.

Column: 70 x 2.5 cm. Flow rate: 12 ml/hour, ⊢—⊣ Fractions collected.

Elution A. Sample: IgA rich whey fraction eluted by buffers 3 and 4 from DEAE cellulose.

Elution B. Sample: Fractions collected from 2 elutions like A concentrated to 2 ml.


A
B
30
50
70
90
70
90
% Transmission
150
200
250
300
Eluant Volume (ml)

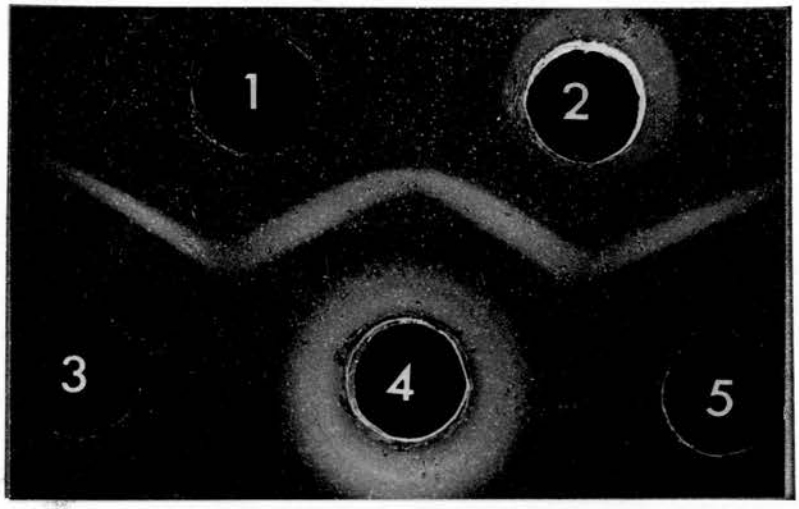


Fig. 7 Comparison of anti-ovine IgA and anti-bovine IgA sera.

1. anti-ovine IgA. 2. anti-bovine IgA. 3 & 5 ovine whey.

4. bovine whey.

obtained in large volumes it was considered likely to be better than whey as a source for isolating IgA.

Therefore globulins, precipitated from 80 ml of lung fluid, were dissolved in 10 ml distilled H_2O, dialysed in 0.01M PO_4 pH 7.6 and applied to DEAE cellulose (Column 20 x 1.5 cm; Flow 25 ml/hour) equilibrated with this buffer (Fig. 8A). After the fall through peak, buffers 3, 4, 5, and 6 were applied and fractions in the last three peaks, which contained IgA were pooled, concentrated and filtered through Sephadex G200. A major peak containing IgA was eluted followed by a smaller IgG shoulder (Fig. 8B). Fractions from the first half of the IgA peak were pooled and concentrated to 2 ml by pressure dialysis with PBS. On gel diffusion and IEP, this material gave a strong precipitin line with anti-IgA identical to one produced by anti-whole serum (Fig. 9). However a very faint line was also detected with anti-IgG on immunodiffusion. This fraction contained 7.5 mg protein/ml and was used as the IgA standard for the single radial diffusion assays.

Preparation of IgG and pre-suckling lamb serum immunoadsorbents.

The method of Porath et al (1967) as modified by Newby et al (1974) was used to prepare IgG and pre-suckling lamb serum immunoadsorbents, which were used for absorbing crude anti-IgA and anti-IgM sera.

Fifteen ml of ice-cold Sepharose 4B was activated by the addition of 10 ml of Cyanogen bromide (CN Br, 50 mg/ml) in a fume cupboard. The pH of the reaction was maintained between 11 and 11.5 by the dropwise addition of Na OH (1 - 10N). After 2 to 3 hours, when the pH had stabilised, the activated Sepharose was thoroughly washed with PBS and packed in a small column (10 x 1 cms). Pre-suckling lamb serum diluted 1 : 10 in PBS or IgG (3 mg/ml) was recycled (20 ml/hour) through such a column for 2 days at 4°C by means of a pump. Unsaturated CN Br ions

were quenched by pumping 0.1M ethanolamine, pH 8.5, through the column for 1 hour and, after a further wash in PBS, the column was ready for use. One to 4 ml of antiserum were applied and fractions forming the fall-through peak were pooled. Antibody - antigen complexes were dissociated by applying 10 ml of 6M urea, 0.2M glycine HCl pH 2.2 to the column and the resulting protein peak was discarded. After another wash with PBS the column was ready for re-use.

Immunodiffusion tests with concentrated fall-through peaks showed that the IgG column could absorb completely the anti-IgG activity of up to 2 ml of either crude anti-IgA or anti-IgM serum, while the pre-suckling lamb serum immunoadsorbent had a capacity of 4 mls for either serum.

Both immunoadsorbents were used frequently over a period of about 9 months either at room temperature or at 4°C without any apparent deterioration.

Discussion

It is not surprising that the IgG preparation consisted of the IgG_1 subclass only, since it is well known that IgG_1 greatly predominates over IgG_2 in sheep whey (Watson and Lascelles, 1973 a). However, as expected, antisera raised against this preparation reacted with IgG_2 as well. No attempt was made to prepare antisera specific for each subclass.

IgG_1 in ruminants has a wide diversity of charge and can exist in polymeric form, properties which make complete separation of it from IgA difficult (Pahud and Mach, 1970; Butler *et al*, 1972; Duncan *et al*, 1972). Therefore since colostrum contains such high concentrations of IgG_1, it is not a suitable fluid from which to purify sheep IgA. Sheep saliva and bovine tears and nasal secretions, fluids containing a high IgA to IgG ratio with little IgM, have each been used as the starting

Fig. 8 Preparation of IgA.

A Fractionation of lung fluid globulin on DEAE cellulose.

Column: 15 x 1.5 cm. Flow rate: 20 ml/hour. Sample: 15 ml.

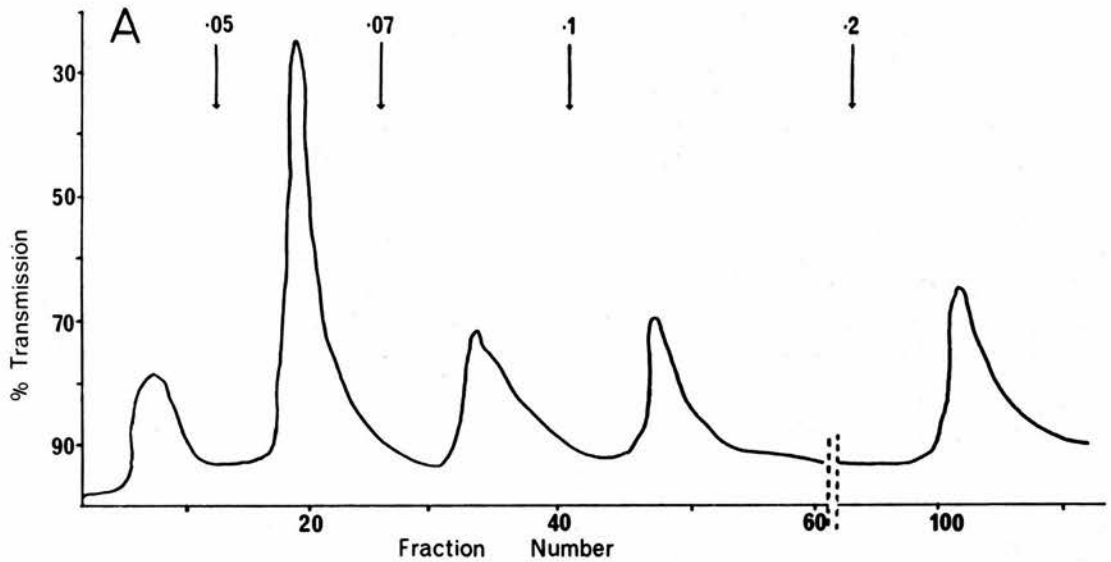
Fraction size: 5 ml.

Buffers: 0.05, 0.07, 0.1 and 0.2 M NaCl all in 0.01 M PO_4, pH 7.6.

B Filtration of IgA rich fractions through Sephadex G 200

Column: 90 x 2.5 cm. Flow rate: 25 ml/hour.

Sample: 2.5 ml concentrate of protein eluted in last 3 peaks of A. ⊢——⊣ = Fractions collected.


A
·05
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100
Fraction Number

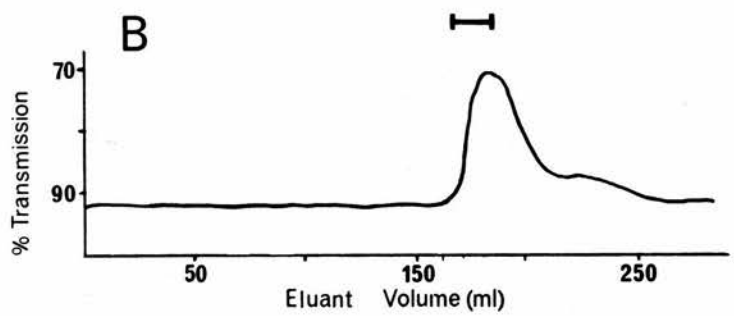

B
70
90
% Transmission
50
150
250
Eluant Volume (ml)

Fig. 9 Immunoelectrophoresis of IgA preparation.

upper well; sheep serum.

lower well; IgA preparation.

upper trough; anti-whole sheep serum.

lower trough; anti-IgA serum.

The anode is on the left.

material in the preparation of IgA (Pahud and Mach, 1970; Morein, 1970; Pedersen and Nansen, 1972). However these fluids are difficult to obtain undiluted in large volumes. If clinical cases are available, it seems that lung fluid from animals with pulmonary adenomatosis is a very useful starting material for isolating sheep IgA.

The IgA and IgM preparations were not completely immunologically pure. However the contaminants must have been present in very low concentrations, since faint lines were only detected on gel diffusion, despite total protein concentrations of 7.5 mg/ml and 3.0 mg/ml for the IgA and IgM preparations respectively.

Some anti-secretory component activity was observed with the anti-IgA serum. However this activity was low and double precipitin rings were not observed in part 2 of this section, when samples were assayed by single radial diffusion with this serum.

PART 2

Immunoglobulins in Ovine Body Fluids.

Introduction

A review of the literature showed that IgG, IgM and IgA had been detected in a number of sheep body fluids (Jonas, 1968 and 1969; Watson and Lascelles, 1971). However the concentrations of these immunoglobulins had only been reported for serum, colostrum, milk and saliva (Pahud and Mach, 1970; Watson and Lascelles, 1973 a and b). As part of this study on the immunity of sheep to PI_3 virus, the immunoglobulin concentrations in certain ovine respiratory secretions were estimated using the specific antisera prepared in part 1. For comparison the immunoglobulin content of serum, colostrum, milk, saliva, lachrymal fluid and bile was also determined.

Materials and Methods.

Animals. Adult sheep of different breeds were sampled.

Serum was harvested from blood collected from the jugular vein.

Nasal secretions were collected by tampons in a manner similar to that described for calves (McKercher et al, 1972). Small tampons+ (2.5 x 0.5 cm) were inserted into the ventral nasal meatus, withdrawn after 10 minutes and squeezed out in a syringe yielding between 0.5 and 1.5 ml of fluid. Tampons were carefully examined for the presence of blood and any doubtful samples were tested by Haemastix*.

Tracheo-bronchial secretions were obtained from sheep lightly anaesthetised with 0.25 ml Immobilon** given intravenously. An endotracheal tube was inserted, and a small piece of tampon attached to the end of a polyethylene catheter was introduced as far as the carina.

+ 'Lil-lets', Southalls (Birmingham) Ltd.

* Ames Co., Miles Laboratories, Slough Bucks.

** Reckitt and Colman, Hull.

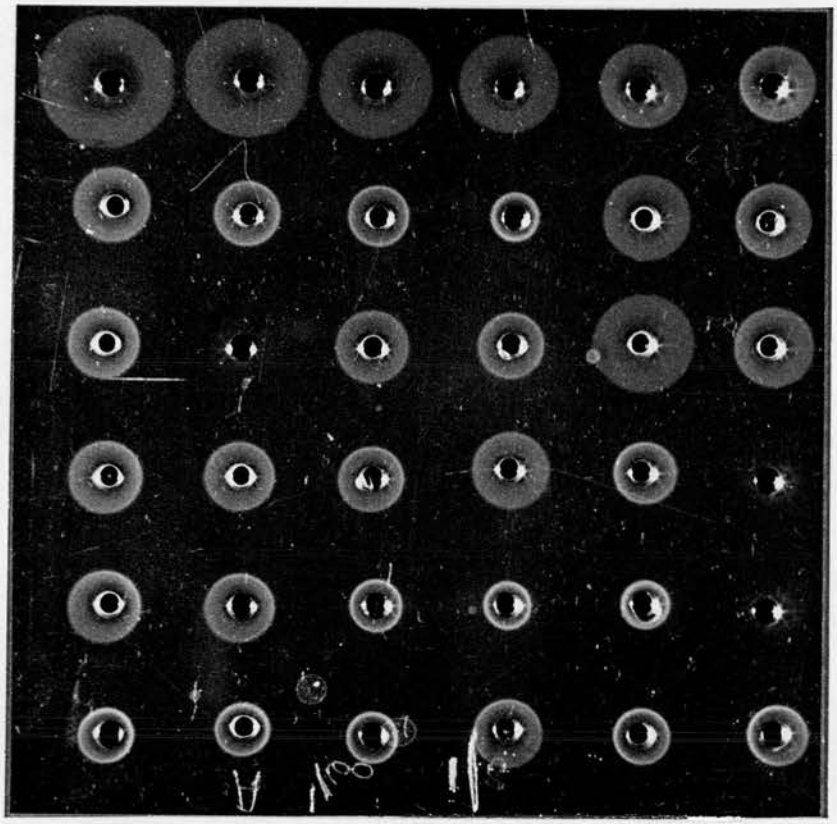


Fig. 10 Single radial diffusion technique.

Agar contained anti-IgG serum diluted 1:40. Standard solutions of IgG were placed in the wells in the top row. All samples producing precipitin ring diameters less than the most dilute IgG standard were assayed again on plates using anti-IgG at 1:100 in the agar.

After 1 minute the tampon was removed and expressed with 0.5 ml PBS in a syringe. Anaesthesia was reversed with 0.25 ml of Revivon+ given intravenously.

Lung fluid was obtained from clinical cases of pulmonary adenomatosis as previously described.

Lachrymal fluid was obtained by placing a small pledge of tampon under the lower eyelid for about 1 minute. The fluid (0.1 to 0.5 ml) was squeezed from the tampon in a syringe.

Saliva was collected by holding small pieces of tampon under the tongue for 1 to 2 minutes.

Bile samples, aspirated from the gall bladder shortly after slaughter, were kindly supplied by Dr. Coop.

Colostrum was milked from ewes before suckling and milk was obtained 21 days after lambing.

Single Radial Diffusion Assay for Immunoglobulins.

The technique of Mancini et al, (1965) as modified by Fahey and McKelvey (1965) was used to estimate IgG, IgA and IgM concentrations.

In preliminary experiments each monospecific immunoglobulin antiserum was incorporated into the agar in a range of dilutions in order to find the highest dilution of serum which would still result in clearly measurable precipitin rings. Thus molten 2% Ionagar No. 2 in 1% sodium azide was mixed with an equal volume of antiserum diluted in buffer (0.2M NaCl, 0.06M $K_2H\ PO_4$, pH 8) held at 56°C. Three ml of each agar solution was poured onto a microscope slide and 5 equidistant 3 mm diameter wells were cut in the agar when it had solidified. Using capillary tubes, the wells were carefully filled to the brim with dilutions of the appropriate standard solutions. Anti-IgG and anti-IgA slides were left to react for 24 hours in humid boxes at room

+ Reckitt and Colman, Hull.

temperature, whereas anti-IgM slides were incubated for 48 hours. Precipitin ring diameters were measured under dark background illumination using a graduated viewer, held against the glass surface of the slide.

Results showed that the optimal dilution of each antiserum in the agar was as follows:- anti-IgG 1/100, anti-IgA 1/60, anti-IgM 1/40. Anti-IgG serum was also used at 1/40 since high concentrations of this immunoglobulin were present in some fluids. Routine estimations of immunoglobulin concentrations were carried out under these conditions, using 20 ml of agar on glass plates (9 x 9 cm) with 36 wells cut 1.2 cm apart in the gel (Fig. 10). Six dilutions of a standard whey, a standard serum or purified IgG were used in each anti-IgA, anti-IgM and anti-IgG agar plate respectively. The ring diameters of the standard dilutions were plotted against their respective concentrations on semi-logarithmic graph paper. A straight line relationship was obtained and the best line of fit was drawn after calculating the regression coefficients. All samples were tested as neat except for serum and colostrum. Serum was diluted 1/20 for IgG, whereas colostrum was diluted 1/60 for IgG and 1/20 for IgA and IgM estimations. The diluent used was PBS.

IgA and IgM concentrations were first expressed as a percentage of the standard whey and standard serum. Later these standards were calibrated to absolute IgA and IgM levels using the preparations described in Part 1. The limits of detection were estimated as; IgG, 1.2 mg/100 ml; IgM 2.2 mg/100 ml and IgA 2.4 mg/100 ml. Samples with protein concentrations below these limits were arbitrarily assigned the value zero.

Although the anti-IgA serum had weak anti-secretory component activity (Section II, part 1), precipitin rings were never observed in concentrated fractions in the 85,000 M.W. region when respiratory

fluids were fractionated by gel filtration (Sections III and IV). Therefore it was considered that the presence of free secretory component in body fluids did not interfere with the IgA estimations.

Results.

1)Quantitation The mean concentrations of IgG, IgM or IgA in each fluid examined are shown in Table 1. IgG, IgM and IgA levels were each expressed as a percentage of the total immunoglobulin in an individual sample. The mean percentages of all samples collected for each fluid are presented in Table 2.

The predominant immunoglobulin in saliva, lung and lachrymal fluid, tracheo-bronchial and nasal secretions was IgA and mean levels exceeded those in serum where IgA was a minor immunoglobulin. Although high levels of IgA were present in colostrum, IgA was a minor component of the total immunoglobulin. IgA was detected at low levels in milk and bile but represented some 10 and 20% of the total immunoglobulin respectively. IgG was present in very high concentrations in colostrum and serum and it was also the major immunoglobulin component in bile and milk. Almost 40% of the immunoglobulin in nasal and tracheo-bronchial secretions was IgG, whereas in saliva, lachrymal and lung fluids the proportion was lower.

Although 10% of immunoglobulin in the serum was IgM, this immunoglobulin was never detected in lung fluid and was found in only a single tracheo-bronchial sample. Low levels of IgM were also present in some milk, saliva, lachrymal fluid and nasal secretion samples. Although actual concentrations were low, IgM made up almost 20% of the bile immunoglobulins - the highest proportion of IgM in all the fluids examined.

2) Fractionation of serum and lung fluid immunoglobulins. Samples of lung fluid and serum obtained on the same day from a sheep affected with

TABLE 1.

IMMUNOGLOBULINS IN CERTAIN SHEEP BODY FLUIDS.

Fluid	Number sampled	Mean protein concentration mg/100 ml (range) ± SE		
		IgA	IgG	IgM
Serum	50	30.9 (8-84) ± 2.7	1882 (1,100-3,000) ± 66.7	201.7 (95-470) ± 12.63
Lachrymal Fluid	14	162.8 (67-250) ± 15.6	26.7 (8-44) ± 3.8	10.21 (0-25) ± 1.36
Saliva	13	93.1 (8-250) ± 21.8	15.8 (6-54) ± 3.3	2.53 (0-9) ± 0.96
Lung Fluid (Pulmonary Adenomatosis)	12	213.8 (55-420) ± 27.32	95.0 (25-200) ± 15.11	0 -
Tracheo-bronchial Secretions	8	42.4 (5-145) ± 17.2	16.7 (5.6-39) ± 4.4	1.0 (0-8) ± 1.0
Nasal Secretions	22	48.7 (18-130) ± 5.8	32.8 (16-52) ± 2.3	5.7 (0-10.2) ± 0.6
Bile	16	7.04 (5-11.6) ±0.43	18.12 (13-28) ± 0.9	7.93 (4.2-8.6) ± 2.96
Milk	14	9.2 (0-28.8) ±2.5	88.8 (47-175) ± 10	4.5 (0-7.4) ± 0.7
Colostrum	19	624 (153-1728) ± 96	10121 (5000-16400) ± 724	291 (82-445) ± 22

TABLE 2.

IMMUNOGLOBULIN CLASS DISTRIBUTION IN CERTAIN SHEEP BODY FLUIDS.

Fluid	Number sampled	Percent Total Immunoglobulin ± SE		
		IgA	IgG	IgM
Serum	50	1.49 ± 0.14	88.64 ± 0.54	9.5 ± 0.42
Lachrymal Fluid	14	81.93 ± 1.75	13.0 ± 1.47	4.69 ± 0.64
Saliva	13	78. 1 ± 3.76	20.23 ± 3.78	1.25 ± 0.6
Lung Fluid (Pulmonary Adenomatosis)	12	70.48 ± 3.52	27.93 ± 3.58	0
Tracheo-Bronchial secretions	8	61.14 ± 4.46	38.21 ± 4.55	0.86 ± 0.86
Nasal Secretions	22	54.0 ± 1.80	39.48 ± 1.84	6.42 ± 0.69
Bile	16	22.83 ± 1.34	58.85 ± 1.60	18.33 ± 1.56
Milk	14	9.34 ± 1.63	84.29 ± 3.12	6.26 ± 1.97
Colostrum	19	5.54 ± 0.61	91.65 ± 0.64	2.79 ± 0.28

pulmonary adenomatosis were filtered through Sephadex G200. The fractions collected were first assayed for immunoglobulins and later each fraction was tested by Mr. Jones for antibody to Mycoplasma ovipneumonia, a common ovine respiratory mycoplasma. A sensitive indirect haemagglutination test was used and the initial titres in both serum and lung fluid were greater than 1 in 4000. In the serum, IgM was eluted before IgG which was ~~much~~ the predominant immunoglobulin, whereas IgA was not detected in any fraction. Antibody activity was found exclusively in fractions containing IgG (Fig. 11).

In the lung fluid, as expected, the major immunoglobulin was IgA, which was eluted before IgG, while IgM was not detected in any fraction. Although some antibody activity was found in fractions containing IgG without IgA, the greatest titres were found in the tubes corresponding to the peak concentration of IgA (Fig. 12).

Two mls of a serum sample which had a high IgA concentration were filtered through Biogel A 1.5M. A protein trace with 2 peaks similar to that shown in Fig. 28 (Section III, part 1) resulted. Eight ml fractions were collected and individually assayed for IgA. IgA was detected only in the fractions eluted between 170 and 230 mls with a peak concentration occurring at about 200 ml. IgA was detected at the same elution volume when whey (Section II, part 1) or nasal or tracheobronchial secretions (Section III, part 1) were filtered through the same column under similar conditions.

Discussion

The concentrations of IgA and IgG in sheep serum approximate closely the values reported by Pahud and Mach (1970), although these authors found lower IgM levels to those reported here. However the IgM levels in cattle sera reported by Mach and Pahud (1971) are also lower than those given by other workers (Duncan et al, 1972; Pederson and Nansen,

Fig. 11 Fractionation of serum from a sheep with pulmonary adenomatosis by filtration through Sephadex G 200.

Column: 90 x 2.5 cm. Flow: 12 ml/hour. Fraction size: 10 ml.

Histogram shows antibodies to Mycoplasma ovipneumonia.

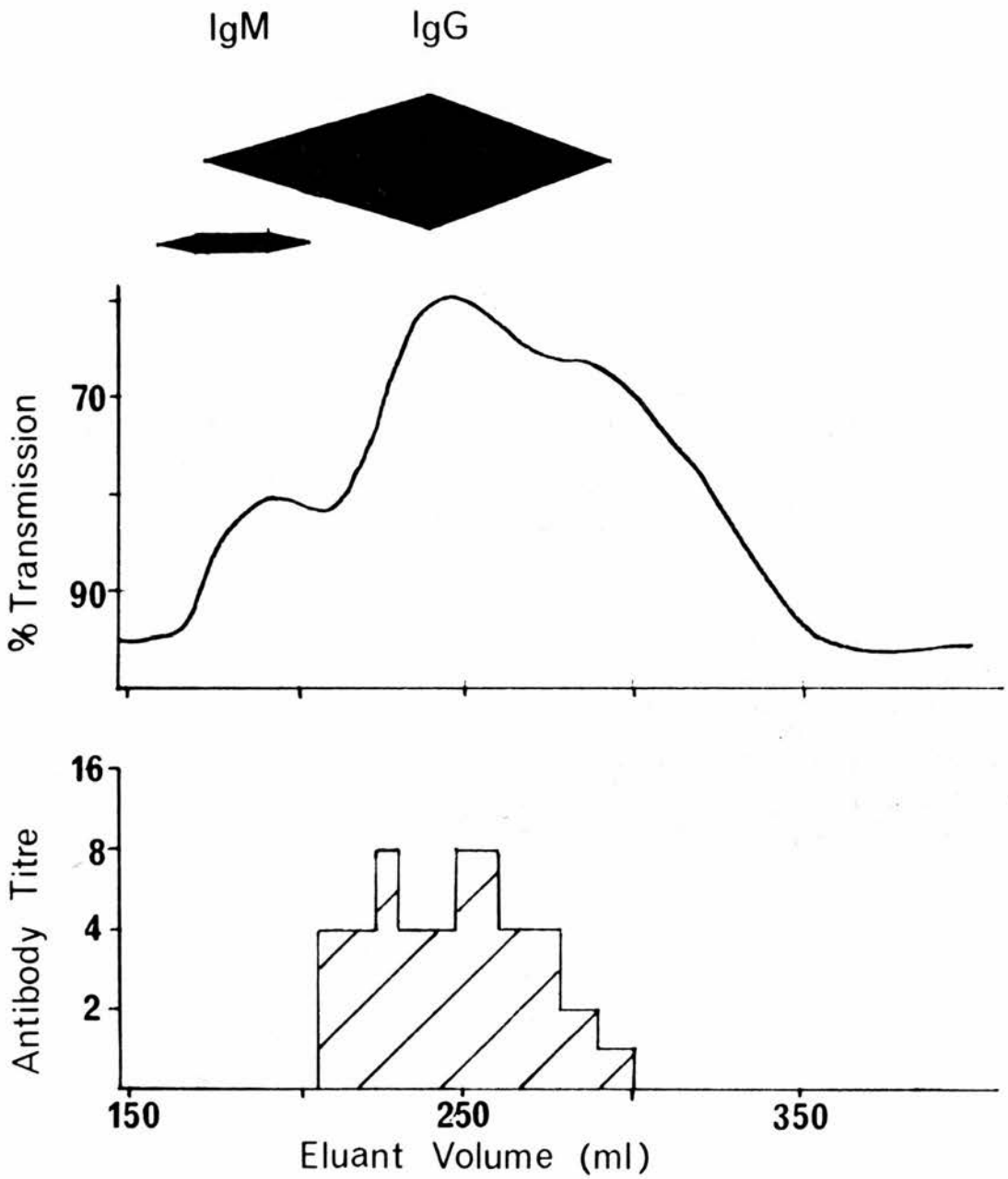
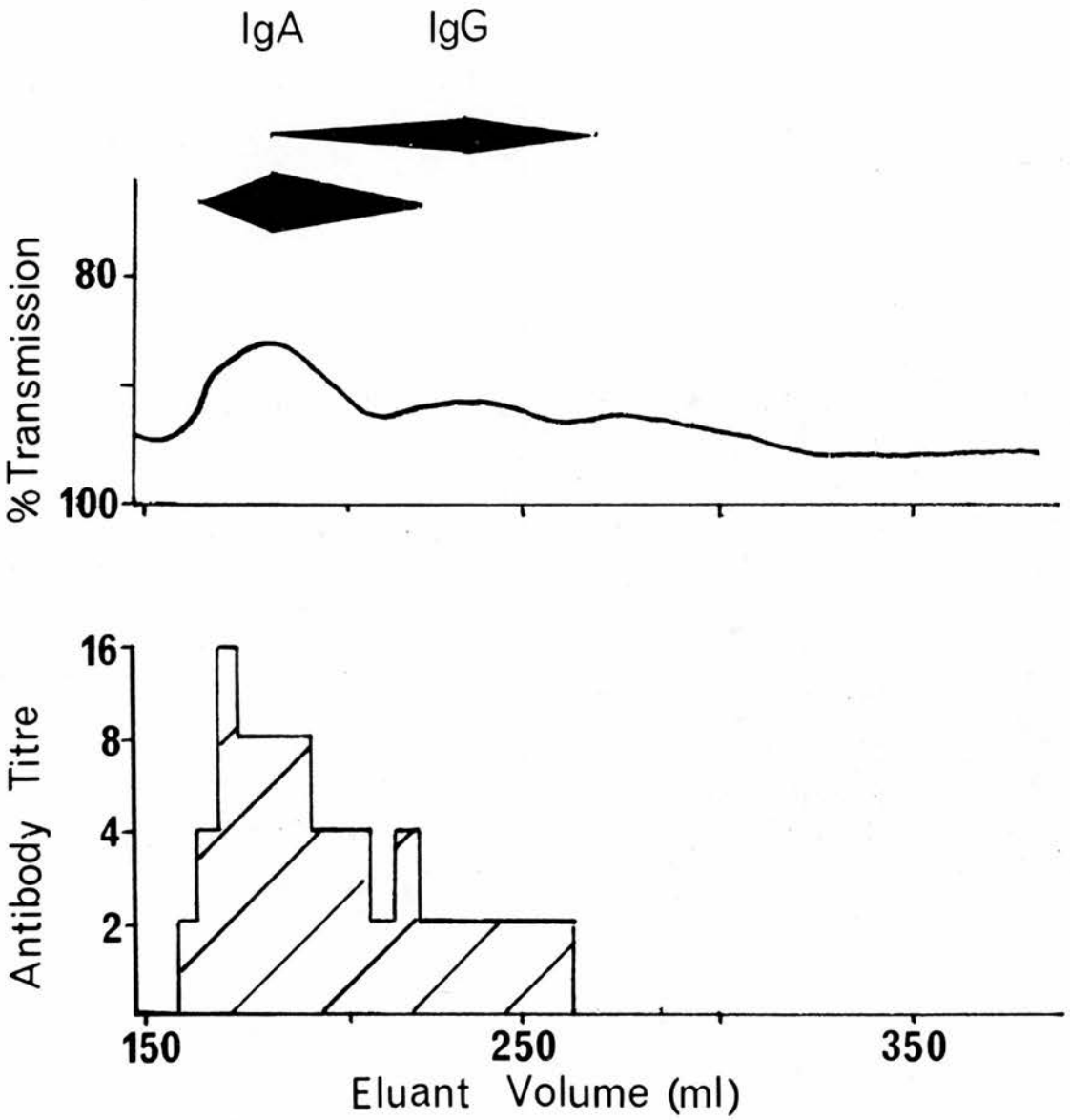

IgM
IgG
% Transmission
70
90
Antibody Titre
16
8
4
2
150
250
350
Eluant Volume (ml)

Fig. 12 Fractionation of lung fluid by filtration through Sephadex G 200.

Column: 90x 2.5 cm. Flow: 12 ml/hour. Fraction size: 10 ml.

Histogram shows antibody titres to Mycoplasma ovipneumonia.


IgA
IgG
% Transmission
80
100
Antibody Titre
16
8
4
2
150
250
350
Eluant Volume (ml)

1972). A considerable variation was noted between sheep in the concentration of immunoglobulins in samples of a particular body fluid, but when concentrations were expressed as a percentage of the total immunoglobulin, the variation was less. Similar variations are recorded in values obtained for cattle, even when adjustment is made in relation to the total protein content of the sample (Pedersen and Nansen, 1972), and the data of Duncan and co-workers (1972) show considerable variation from week to week in samples taken from the same animal. Later measurements (Section III, part 1) showed a similar situation in sheep nasal secretions. When these factors together with variables such as sampling technique and time after parturition are taken into account, the values reported here for ovine saliva, milk and colostrum are similar to the findings of others (Pahud and Mach, 1970; Watson and Lascelles, 1973 a & b).

The immunoglobulin levels found here in sheep lachrymal fluid are similar to results reported for the bovine (Pedersen and Nansen, 1972). In accord with the findings of Mach and Pahud (1971) in cattle IgM constituted a relatively high proportion of bile immunoglobulins. Sheep nasal secretions appeared to have a somewhat lower IgA to IgG ratio than cattle (Duncan et al, 1972), although the possible effect of a different sampling method was not ruled out. Tracheo-bronchial secretions from normal sheep and lung fluid from sheep with pulmonary adenomatosis had similar proportions of IgA and IgG with virtually no detectable IgM.

The very high concentration of IgA in the lung fluid suggests that most of the immunoglobulin is present in the fluid when it is in situ in the bronchioles and alveoli and it is unlikely that the flushing of the upper respiratory mucous surfaces by the lung fluid during collection contributes significantly to the immunoglobulin

content.

Gel filtration showed that serum IgA was larger than IgG and had a similar molecular size to IgA in the secretions. Others have estimated the molecular weight of sheep serum IgA at 350,000, which is larger than IgG but smaller than secretory IgA (Pahud and Mach, 1970; Beh et al, 1974).

The results confirmed previous findings that IgA is the major immunoglobulin in sheep saliva (Pahud and Mach, 1970; Watson and Lascelles, 1973 b). A similar situation was found in lachrymal fluid and nasal secretions. The much higher IgA to IgG ratio in these fluids compared with serum proved that local production, selective transport, or a combination of these mechanisms must operate for IgA at these mucous surfaces. In addition, the finding that most of the lung fluid antibody to mycoplasma was associated with IgA, suggested that IgA antibodies in the respiratory secretions might have a protective role in sheep respiratory diseases.

S E C T I O N III

Introduction

The immunoglobulin studies in Section II showed that an IgA system was present in ovine respiratory secretions and that IgA antibodies to at least one respiratory micro-organism could be detected in lung fluid collected from cases of pulmonary adenomatosis. This section describes 4 experiments designed to study the immune response of lambs to PI_3 virus with particular reference to the characterisation and role of antibody in the respiratory tract.

The first part of this section describes a study of the immune response of 9 month old lambs following infection with PI_3. In addition to antibody and immunoglobulin assays in the serum, nasal and tracheo-bronchial secretions, an attempt was made to detect cell mediated immunity to the virus using an intradermal hypersensitivity test.

Three experiments carried out with specific pathogen free (SPF) lambs are described in the second part of the section. In these experiments the nasal and serum antibody response was measured after single or combination inoculations of live or inactivated PI_3, administered either IN or IM. The protective effect of each type of 'vaccination' was assessed by challenging the lambs with an aerosol of live virus. The term 'vaccination' will be used throughout to include primary exposure to live virus, although this was not attenuated in any way. For ease of group to group comparisons the groups of lambs in the 3 separate experiments are numbered sequentially from 1 to 12.

Materials and Methods

Techniques common to this section and Section IV are mentioned here. Other procedures and techniques relating only to a particular experiment will be described with that experiment.

Lambs. Nine month old conventionally reared Scottish Half Bred hoggs were used in part 1 of this section. The animals were housed in the isolation building, when it was not occupied by SPF lambs. The lambs were fed commercial concentrates and hay ad lib. The groups were housed in separate rooms, which as usual were pressure ventilated with filtered air. Personnel attending the animals wore sterile clothing and moved from the control to the infected group.

Hysterectomy derived, colostrum deprived lambs reared in conditions sufficient to maintain their pathogen free status were used in the experiments described in part 2 of this section (Hart et al, 1971). The animals were fed on sterilised diets of diluted condensed cows' milk and chopped hay. Precautions were taken to reduce the risk of cross contamination of virus between groups of lambs. Thus personnel always moved from rooms containing control lambs to rooms containing infected lambs and then to the outside of the isolation building. They then showered and changed into fresh sterile clothing before re-entering the animal rooms.

Erythrocytes. Guinea pig blood was collected by cardiac puncture and stored in Alsever's solution at $4^{o}C$. The erythrocytes were washed three times in PBS and, after the packed cell volume had been determined in a haematocrit tube, the cell suspension was adjusted to the appropriate concentration with PBS. Chicken erythrocytes, from wing vein blood, were prepared in a similar manner.

Tissue Cultures and Maintainance Medium. Monolayers of secondary foetal lamb kidney cells (FLK) in tubes or Roux flasks were kindly supplied by Miss Anderson and Mrs. Hood. The cells were maintained with medium 199 supplemented with 2% horse serum, 200 iu/ml penicillin and 100 μg/ml streptomycin.

Virus. The G2 isolate of ovine PI_3 (Hore, 1966) was used throughout

these experiments at the sixth passage. The virus was propagated on secondary FLK monolayers maintained with medium 199.

Preparation of Virus Pools. After removal of the growth medium, FLK monolayers in Roux flasks were washed twice with Hank's basic salt solution and incoulated with 2 ml of fluid containing the virus. The virus was left to adsorb for 30 to 60 minutes at 37°C before 50 ml of medium was added to each flask. After 5 or 6 days' incubation at 37°C, when a widespread cytopathic effect was observed, the flasks were frozen and thawed twice to disrupt the cells and the culture fluid harvested. Pools of infective fluid were centrifuged (1,500 g, 20 minutes) to remove the cellular debris and stored in aliquots at -70°C until required.

Infectivity Titrations. Ten-fold serial dilutions of virus were made by pipetting 0.2 ml of infective fluid in 1.8 ml PBS containing 1% bovine serum albumin and 0.2 ml of each dilution was inoculated into each of 4 to 6 FLK monolayers in tubes. Virus was left to adsorb for 1 hour at 37°C before 1 ml of maintenance medium was inoculated into each tube. After 4 or 5 days' culture at 37°C, each tube was read by the haemadsorption technique. The 50% end point was calculated (Reed and Meunch, 1938) and the infectivity was expressed as the number of tissue culture infective doses per ml (TCID 50/ml).

Haemadsorption. This test was used to detect the presence of PI_3 in cell monolayers. Medium was removed from the tissue culture tubes and 1 ml of 1% washed guinea pig red cells was added to each monolayer. After about 30 minutes at room temperature, non-adherent red cells were removed by 2 washes with PBS. The monolayers were then examined for the presence of haemadsorption.

Virus inactivation. Pools of virus were inactivated with 0.2% formalin (40% formaldehyde solution) for 72 hours at 26°C. Inactivation was

checked by inoculating 1.0 ml of this fluid into each of 15 FLK monolayers in tubes. After 1 week, the supernate fluid from these tubes was passed to further FLK cultures and 7 days later these were tested by haemadsorption for the presence of virus. If no haemadsorption was detected the virus was considered to have been inactivated.

Freund's complete adjuvant (FCA) was made up as described in Section II.

Virus Isolation. Nasal swabs were taken from the right nasal passage of each lamb and were placed immediately into 3.0 ml of ice-cold transport medium (Hank's balanced salt solution with 2% bovine albumin, 600 iu/ml penicillin and 300 μg/ml streptmycin). Two-tenths of an ml of this fluid was inoculated onto each of 4 FLK monolayers in tubes within 2 hours or else the fluid was stored at -70°C until use. After 30 minutes at 37°C, one ml of maintenance medium was added to each tube. The cultures were read by haemadsorption after 6 or 7 days' incubation at 37°C.

Serum and nasal secretion samples. These were collected as described in Section II part 2. It was found that tampons cut to approximately 0.5 cm x 0.5 cm were an appropriate size for lambs less than 4 weeks old, while 1.0 x 0.5 cm tampons were used in older animals. Nasal secretions were collected from the left nostril (not the side swabbed for virus isolation), examined for blood and stored as previously described for adult sheep.

Immunoglobulin assays. IgA, IgG and IgM concentrations were estimated as described in Section II, part 2. Most SPF lamb sera were tested undiluted for IgG.

Virus Neutralisation Test. A microneutralisation test was employed to allow large numbers of sera and secretions to be assayed for antibody to PI_3. Similar tests have been used for detecting neutralising antibody in the bovine (Rossi and Kiesel, 1971; McKercher et al, 1972).

One drop (0.025 ml) containing 16 TCID 50 (Range 8 - 32) of virus was added to an equal volume of 2 fold dilutions of heat inactivated (56°C for 30 minutes) serum or nasal secretions made in duplicate or triplicate in flat-bottomed microplates.* One drop (0.025 ml) of medium was used as the diluent and the dilutions were made by means of microdiluter* loops with 0.025 ml capacity. The medium used was modified Eagle's minimal essential medium supplemented with 5% heat inactivated foetal bovine serum, 1% 1.0M magnesium chloride, 200 iu/ml penicillin, 100µg/ml streptomycin and 50 units/ml mycostatin. Serum-virus mixtures were held at room temperature for 90 minutes before approximately 14,000 FLK cells in 0.1 ml medium were added to each well. The plates were then sealed with tape to prevent CO_2 loss and incubated at 37°C. A confluent monolayer was obtained by 48 hours. On the fourth day of culture the seals were removed and 0.025 ml of 2% guinea pig red cells was added to each well. After 30 minutes the plates were washed with PBS to remove non-adherent red cells, excess PBS was then removed and the plates were observed for haemadsorption. Neutralisation was taken as occurring in those cups where no haemadsorption was detected and neutralising titres were calculated by the method of Reed and Meunch (1938). Control positive and negative sera, titrations of test virus and tests for the cytotoxicity of the samples were included in each test. All titres in the text are expressed as reciprocals.

At first some tests were done using U-bottomed microplates. These plates have the advantage that the erythrocytes sediment to form a button in the wells so that the end point of the test can be read by haemagglutination. However it was found that reading the end point by this method was more subjective.

*Cook Instruments, Flow Laboratories, Irvine, Ayrshire.

Haemagglutination (HA) and Haemagglutination inhibition (HI) Tests. For PI_3 Virus. A microtitre version of methods similar to those employed by Hore (1968) were used.

Virus pools, prepared as described above, were used as the source of haemagglutinin. HA activity was titrated in plastic microplates with U-shaped wells using PBS as a diluent. Doubling dilutions of virus were made with the diluters as described for the microneutralisation test, before 0.025 ml of a 0.5% suspension of guinea pig erythrocytes was added to each cup. Plates were held for $1\frac{1}{2}$ hours at room temperature before the end points were read. The titre was taken as the highest dilution of haemagglutinin to cause complete agglutination of erythrocytes and this dilution was considered to contain one HA unit per 0.025 ml.

All samples to be tested for HI antibody were inactivated at 56°C for 30 minutes. Aliquots of sera (0.2 ml) were diluted 1/10 in PBS and absorbed with a 10% suspension of guinea pig erythrocytes, to remove any non-specific agglutinins. Due to the low antibody titres and small volumes of sample available, various fractions and nasal secretion samples were not absorbed with erythrocytes. Therefore control samples without virus were always included in each test, but non-specific agglutinins were never seen.

For antibody titrations serial 2-fold dilutions of each test serum were mixed in microplates with equal volumes (0.025 ml) of a dilution of virus containing 4 HA units. After 1 hour at room temperature, 0.025 ml of a 0.5% suspension of guinea pig erythrocytes was added to each well. The plates were held at room temperature for $1\frac{1}{2}$ to 2 hours before the end point was read. For sera this was taken as the highest dilution which completely inhibited HA, whereas for the nasal secretions and fractions a 50% end point was used, in order to increase the

sensitivity of the test.

Each test included a HA titration of the test virus and known positive and negative serum titrations as well as erythrocyte controls.

For Newcastle Disease Virus (NDV). Formalinised (0.2%) NDV haemagglutinin (Hitchner B1 strain) was kindly supplied by Dr. S. McNulty (Royal Dick Veterinary School, Edinburgh). An HI test was carried out using 4 HA units in the way just described for PI_3 except that a 1% suspension of chicken erythrocytes was used. Since these cells sedimented more rapidly than guinea pig erythrocytes the end point, taken at 50% HA, could be read after 30 minutes.

For Parainfluenza type 2 (PI_2) virus. Human PI_2 virus haemagglutinin was kindly supplied by Mr. Sharp. The virus had been passed 3 times in Hep II cells after being supplied as a reference strain by the Central Public Health Laboratory, London. Harvest material, diluted in PBS to contain 4 HA units per 0.025 ml, was used in the HI test, which was carried out using guinea pig red cells in the way described for PI_3.

For Parainfluenza type 1 (PI_1) virus. Sendai virus was obtained from Mr. Scott. The virus had been propagated in eggs and was diluted in PBS to contain 4 HA units per 0.025 ml. The HI test was carried out in the manner described for PI_3.

Treatment for non-specific inhibitors. Certain nasal secretion samples and fractions were treated with receptor destroying enzyme (RDE)* in an attempt to remove non-antibody inhibitors of HA. Three drops of RDE were dispensed into each cup forming the top row of a U-bottomed microtitre plate and one drop (0.025 ml) of each sample under test was added to these wells. The plate was sealed with tape and incubated overnight at 37°C, after which it was heated for 1 hour at 60°C in a shallow water bath to destroy residual activity of the enzyme.

* Extracted from Vibrio cholerae , Lot K7892 Wellcome.

The seal was then removed and doubling dilutions of each sample were made in PBS using the microdilutors. Omitting the top row containing the RDE, virus and erythrocytes were dispensed as before and the test was read taking the dilution of the test sample in the second top row at 1/8.

Fractionation of Serum and Nasal Secretions. Initial experiments showed that a better separation of the 3 immunoglobulin classes was achieved using filtration through Biogel A 1.5M compared with Sephadex G200, although G200 gave a better resolution of lower molecular weight molecules (compare Figs. 19 and 28 with Figs. 11 and 12). Therefore fractionation of serum and secretions was carried out by filtration through an upward flowing column (70 x 2.5 cm) of Biogel A 1.5M in PBS kept at + 4°C. PBS was pumped through the column at 12 ml/hour and 8 to 12 ml fractions were collected. Neutralising and HI antibody determinations were made on the fractions, usually after concentration by dialysis in solid polyethylene glycol (20,000 MW). In order to increase the sensitivity of the test, 50% inhibition of HA was read as the end point in the HI assay. IgA, IgG and IgM were detected in the fractions either by SRD or by double diffusion using the specific antisera.

Aerosol apparatus and Challenge Procedures. The first apparatus built for exposing lambs to aerosols is shown diagramatically in Fig. 13. Aerosol, produced by a Wright nebuliser* operating at 10 pounds per in^2 from a compressed air cylinder, was directed into a 22 l. airtight steel reservoir. Under these conditions about 0.1 ml of culture fluid was nebulised each minute producing 8 l. of aerosol which, according to the specifications of the atomiser, had a particle size less than 10μ diameter. The aerosol cloud was withdrawn from the reservoir through a

* Aerosol Products (Colchester) Ltd.

Fig. 13 Diagram of Aerosol Apparatus.

Key.

N = nebuliser

R = reservoir

T = tap

V = non-return valve

M = mask

F_2 = low resistance filter

MN = manometer

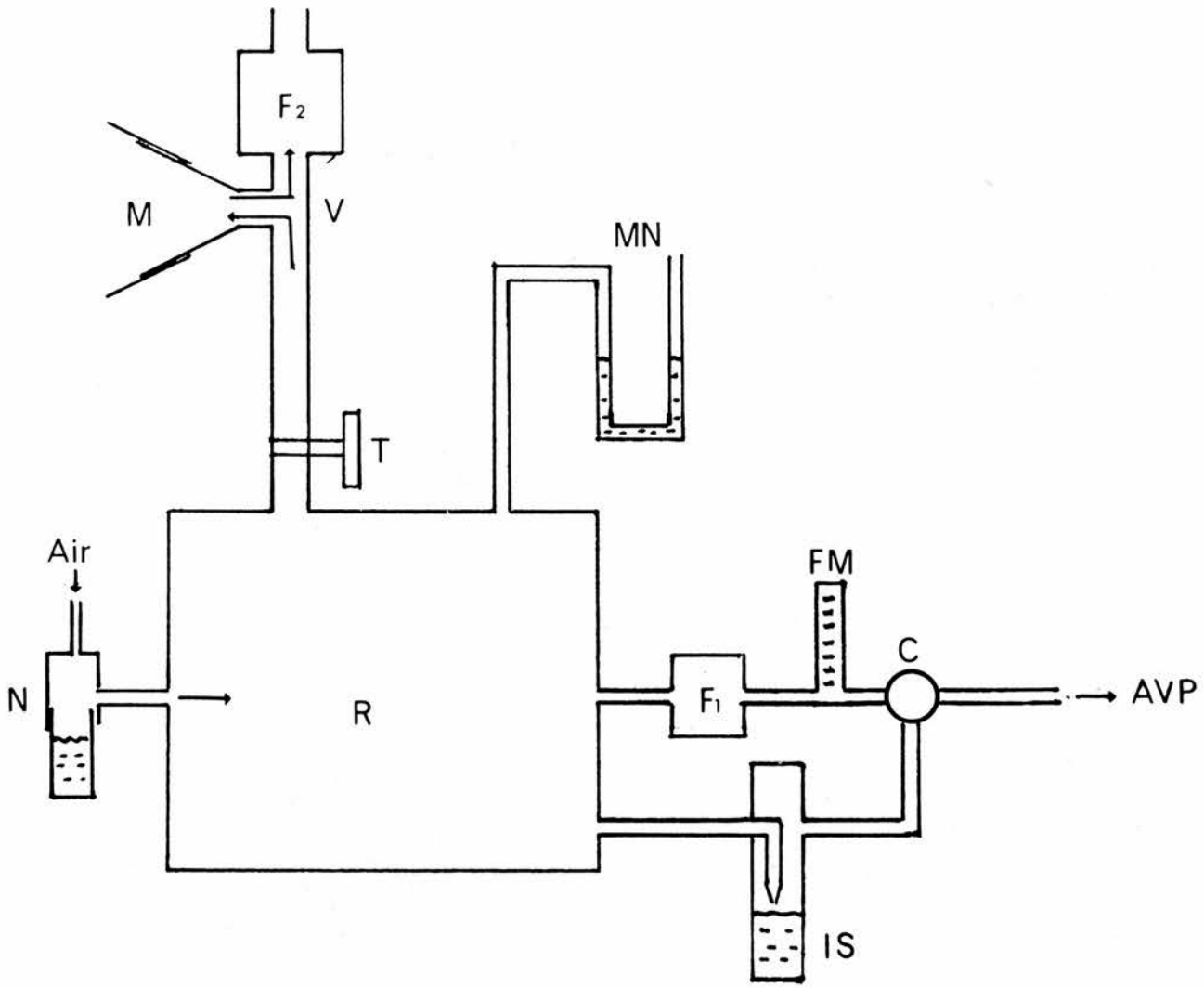
F_1 = filter

FM = flow meter

C = 3 way cock

IS = impinger sampler

AVP = adjustable vacuum pump


F2
M
V
MN
T
Air
N
R
FM
C
F1
AVP
IS

filter and flow meter by a vacuum pump adjusted so that the pressure inside the chamber was atmospheric. After a few minutes, to allow conditions inside the reservoir to stabilise, each lamb was restrained in a sheep cradle and made to inhale for 10 minutes from a mask sealed round its muzzle. The mask used was a small inhalation anaesthetic cone, which had been extended by a 2 in. strip of polythene glued around the rim of its wider opening. The polythene strip was held round the lamb's muzzle with a strong elastic band to form a crude seal. The mask was connected to the reservoir by a 2.5 cm diameter tube and a non-return valve ensured that exhaled air passed to the atmosphere through a low resistance filter. During the exposure period the flow through the exhaust pump was reduced, by a rate considered equal to the lamb's minute volume, so that the pressure in the reservoir remained atmospheric. A 5 minute sample of the aerosol cloud was obtained when a lamb was not being challenged, by diverting the exhaust flow through an impinger* containing 30.0 ml of transport medium with 0.1% anti-foaming agent. An aliquot of the medium was titrated for virus infectivity and hence, knowing the volume of aerosol passing into the fluid, the infectivity of each litre of aerosol could be calculated. The dose of virus (D) inhaled by each lamb was derived from the formula $D = I \times M \times T$, where I is the infectivity per litre of aerosol, M is an estimate of the lamb's minute volume in litres and T is the period of exposure in minutes.

A preliminary trial of this apparatus was carried out in conjunction with Dr. N. Gilmour. A 7 week old conventionally reared lamb was exposed for 10 minutes to an aerosol of Pasturella haemolytica, nebulised from a broth culture containing approximately 1.1×10^9 viable bacteria per ml. An impinger sample was collected and 20 minutes later the lamb was

* All Glass Impinger. Millipore (U.K.) Ltd.

killed and the lungs removed. One gm pieces from each lobe were ground up with 5 ml of broth and dilutions of the lung suspension were titrated and cultured overnight on blood agar plates. The number of viable bacteria in each lung suspension sample ranged between 35 and 425 colony forming units/ml, while a total of 2×10^6 viable cells were estimated in the impinger fluid. These results indicated that P. haemolytica remained viable in the aerosol and that large numbers of viable particles were penetrating deep into the respiratory tract. In another trial tissue culture fluid containing $10^{4.3}$ TCID 50 PI_3 per ml was nebulised and $10^{1.8}$ TCID 50 was recovered in the impinger fluid, showing that PI_3 was also viable in the aerosol.

This apparatus was used for challenging the SPF lambs in Experiment 1. An aliquot of the virus pool used for vaccination, containing 10^6 TCID 50/ml, was nebulised. The minute volume of the lambs was estimated as 3 l. and, from the titre of the impinger fluid, it was calculated that each lamb inhaled a total of $10^{4.9}$ TCID 50 of virus.

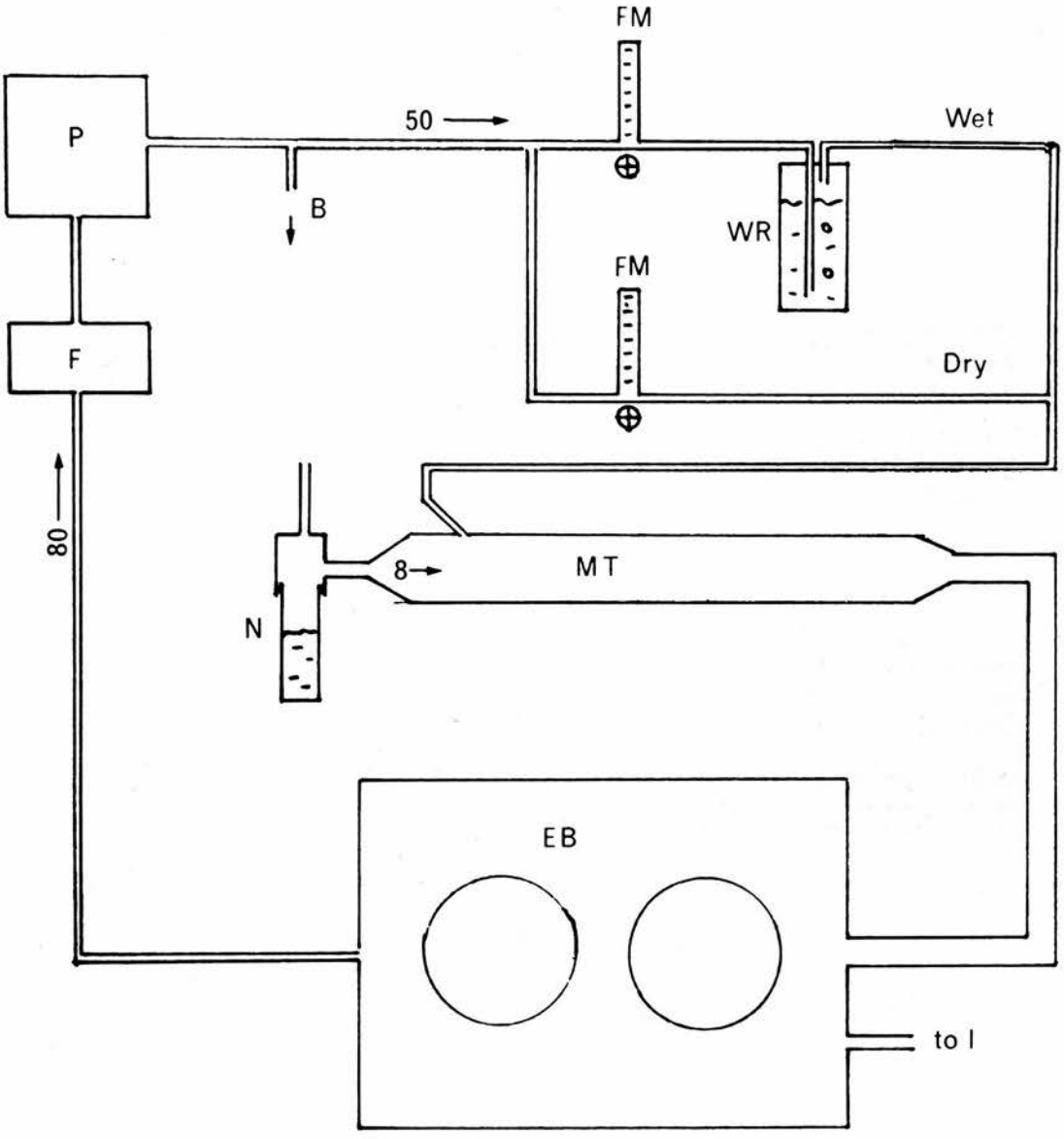
This apparatus was used successfully in Experiment 1 and by others in experiments with P. haemolytica and with adenovirus. However lambs could only be exposed individually to the aerosol, a time consuming procedure. Therefore, before Experiments 2 and 3, the apparatus was modified so that 4 lambs could be exposed simultaneously (Figs. 14 and 15). Eight l/minute of aerosol, produced as before, were mixed with 50 l /minute of air from a compressor-vacuum pump and passed into a 0.3 m^3 exposure box with four 15 cm diameter holes. The relative humidity of the mixing air could be increased above the ambient level by controlling the proportion of it bubbling through a reservoir filled with water. The humidity of the aerosol cloud was monitored by a hygrometer in the exposure box. Each lamb's head was passed through one

Fig. 14 Diagram of modified aerosol apparatus.

Key.

P = pump	MT = mixing tube
FM = flow meter	EB = exposure box
WR = water reservoir	I = impinger
N = nebuliser	F = filter

Figures indicate the flow rate in litres/minute.


FM
P
50
Wet
B
WR
FM
F
Dry
80
8
MT
N
EB
to I

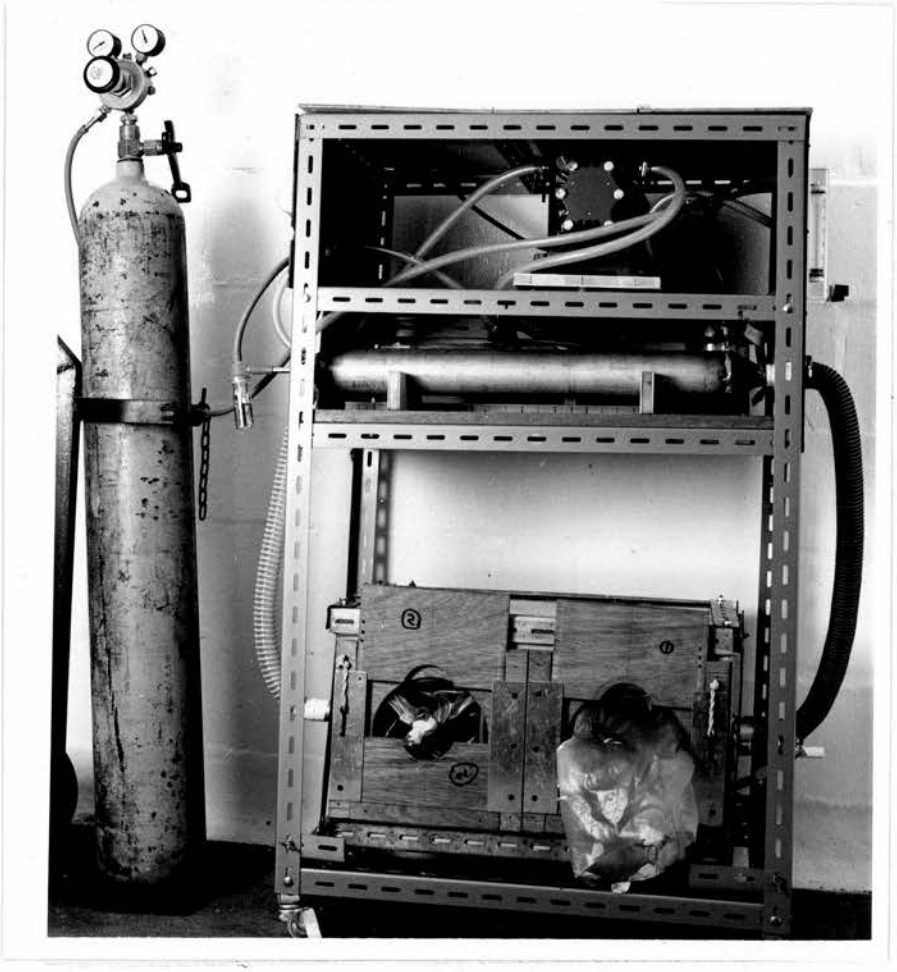


Fig. 15 Aerosol Apparatus used in Experiments 2 and 3

hole in the exposure box and restrained inside this chamber by a yoke and a polythene sleeve, which was taped round the animal's neck to form a seal. Aerosol leakage was further reduced by maintaining an extraction rate of 80 l /minute from the box. The four lambs in each group were exposed simultaneously for 30 minutes. Ten minute impinger samples were taken midway through certain exposure periods in each experiment. In Experiment 2 tissue culture fluid containing $10^{8.3}$ TCID 50 of virus/ml was nebulised and it was estimated from the formula, assuming a minute volume of 3.1, that each lamb inhaled $10^{5.3}$ TCID 50 of virus. Fluid containing $10^{7.0}$ TCID 50/ml was nebulised in Experiment 3 but virus was not recovered in the impinger sample, a finding which is discussed in the description of that experiment.

Before and after use both sets of aerosol equipment were disconnected and left to stand overnight in an atmosphere of formaldehyde vapour, produced by adding formalin to potassium permanganate crystals. In order to remove any traces of this gas, air was pumped through the apparatus for 30 minutes before the lambs were exposed. Personnel operating the aerosol equipment always wore protective respirators.

Necropsy Procedure. The lambs were shot with a humane killer and exsanguinated by severing the vessels in the neck.

Histological Procedures. Blocks from representative areas of lung, bronchus, trachea and nasal septum were fixed in 10% formol saline with secondary fixation in mercuric chloride. Five μ paraffin sections were cut and, after processing, stained with celestin-blue haematoxylin and eosin according to Culling (1963). Some sections were stained with Pollack's trichrome (Pollack, 1944).

PART I

Immunoglobulins, Antibodies and Inhibitors of Parainfluenza 3 Virus in the Respiratory Secretions of Sheep.

Introduction

The results from Section II indicated the presence of an IgA antibody system in the sheep respiratory tract. The object of the experiment about to be described was to determine whether IgA specific antibody was produced in the respiratory secretions of lambs after infection with PI_3. Attempts were also made to discover if a delayed-type hypersensitivity skin reaction could be elicited following infection with this virus.

Experimental Procedures

Eight 9 month old Scottish Half Bred lambs, which were seronegative for PI_3 antibody were randomly placed into 2 groups of 4. The animals were housed and fed as described in 'Materials and Methods'.

One ml of fluid containing 10^8 TCID 50 of PI_3 was instilled into each nostril of the lambs in one group. Lambs in the remaining, control group each received the same volume of tissue culture fluid by the same route. All lambs were swabbed daily for 1 week after inoculation and clinical signs were noted during this period. Pre-inoculation blood and nasal secretion samples were collected and thereafter at 2 to 5 day intervals for 5 weeks. Tracheo-bronchial secretions, obtained as described in Section II, were collected from all sheep before inoculation and 4,9, 18 and 23 days after inoculation. Samples of each fluid were tested for HI and neutralising antibody to PI_3. The nasal secretions were assayed for immunoglobulin content, and pools of nasal and of tracheo-bronchial secretions were fractionated by gel filtration.

Virus Antigen. Virus from 60 ml of fluid identical to the inoculum was pelleted by ultracentrifugation (54,000 g, 2 hours). The pellet was

resuspended in 2 ml of PBS and heat inactivated at 56°C for 10 minutes (Hore, 1968). Control fluid from uninfected cultures maintained in parallel was treated to all procedures in a similar way. The test antigen contained 2560 HA units of virus/ml.

Intradermal test. An area about 6 inches square was clipped bare over the lateral aspect of the thorax of each lamb and four smaller squares were marked on the skin with a pen. Either 0.1 ml of antigen or 0.1 ml of control fluid was injected intradermally within each delineated square of skin. Thus each animal received 2 adjacent intradermal injections of both virus antigen and control fluid. The skin thickness was measured with calipers at each inoculation site before, and 24, 48 and 72 hours after inoculation.

RESULTS

A muco-purulent nasal discharge, observed 3 to 6 days after inoculation with virus, was the only clinical abnormality noted. This sign was not observed in the control group. One of the control lambs died on day 3 while anaesthetised for tracheo-bronchial secretion sampling.

Virus was recovered on at least 2 occasions from nasal swabs taken from each lamb in the infected group (Table 3) and was never recovered from the control lambs.

HI antibody was first detected in the sera 7 to 9 days after the lambs were inoculated with virus and neutralising titres were found about 2 days later (Fig. 16). Thereafter serum HI and neutralising titres correlated closely except in lamb 882 in which an increase in neutralising antibody, without a concomitant HI rise, was observed on days 32 and 36. Antibody was never detected in the sera of the 3 surviving control lambs.

Pre-inoculation nasal secretions gave HI titres ranging from 8

TABLE 3.

Virus Recovered from Nasal Swabs.

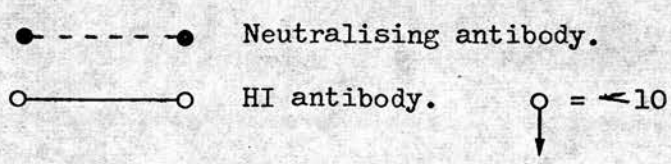
Group	Lamb No.	Days after inoculation 1	2	3	4	5	6	7
Infected	866	-	-	-	-	-	+	+
	882	-	-	-	-	+	+	+
	885	-	-	+	+	+	+	-
	894	-	-	-	-	+	+	-
Control	870	-	-	-	-	-	-	-
	886	-	-	-	-	-	-	-
	887	-	-	-	-	-	-	-
	847	-	-	-	D			

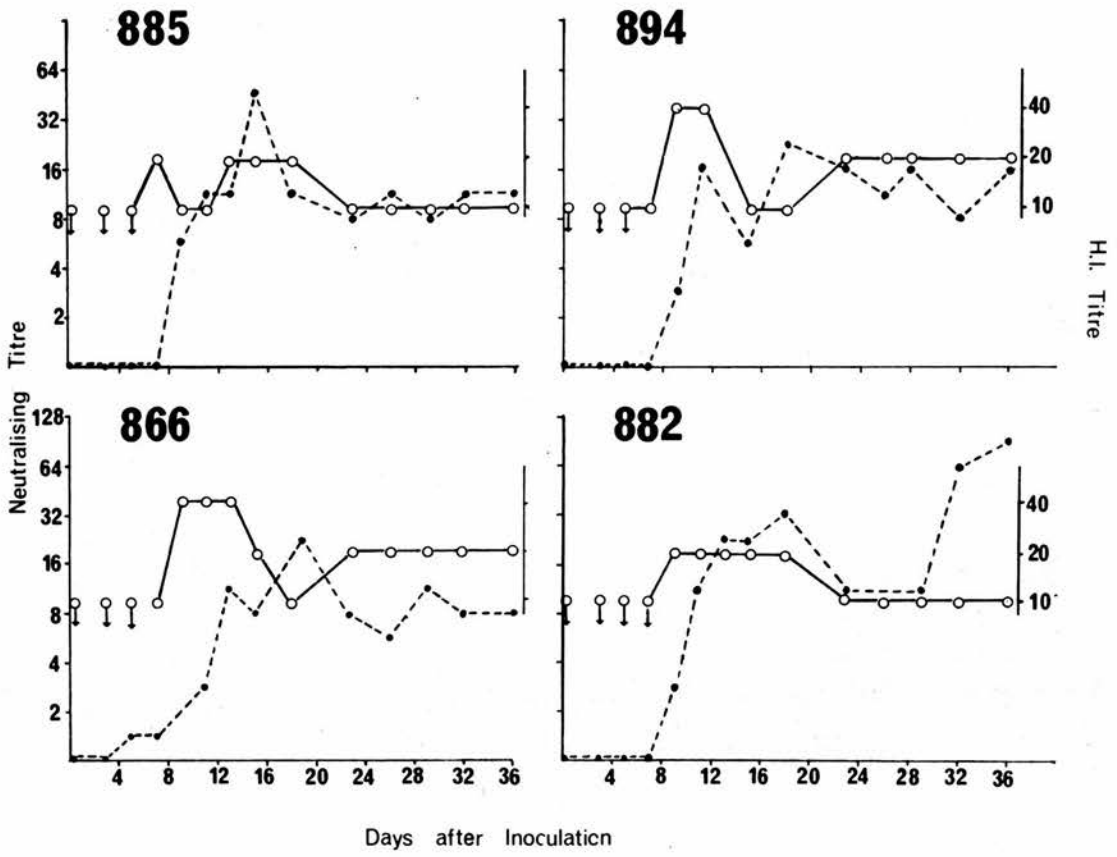
\+ virus recovered

\- no virus recovered

D animal died

Fig. 16 Serum neutralising and HI titres in individual lambs after infection with PI_3.




885
894
866
882
Neutralising Titre
H.I. Titre
64
32
16
8
4
2
128
40
20
10
4
8
12
16
20
24
28
32
36
Days after Inoculatien

to 32. After RDE treatment the titres were reduced in 7 lambs to 8 and to less than 8 in one lamb. After infection no significant (i.e four-fold) rise in HI activity was evident (Table 4).

To check for the possibility of HI activity in the tampon itself, PBS, in which tampons had been immersed for 5 minutes and then expressed, was tested and found to have no HI activity.

Pre-inoculation nasal secretion neutralising titres were less than 2 and remained undetectable in the control lambs throughout the experiment. In the infected group nasal secretion neutralising antibody was detected by day 5 in 3 lambs, whereas in lamb 866 significant titres were not recorded before day 18 (Fig. 17). Individual peak nasal secretion titres ranged between 11 and 23. Nasal antibody persisted for 4 weeks or more, although detection was sporadic towards the end of the experiment.

Low neutralising titres were found in the tracheo-bronchial secretions of 2 lambs 9 days after infection with no neutralising activity detectable in the control or pre-inoculation samples. However HI activity, at titres ranging from 4 to 16, was present in all the samples.

The IgA, IgG and IgM concentrations found in control and infected lambs' nasal secretions showed marked variation from day to day (Figs. 17 and 18). IgA predominated over IgG in all but 3 samples, in which slightly higher IgG concentrations were recorded. Low levels of IgM were present in most of the samples. No difference in nasal secretion immunoglobulin levels was found between the control and infected lambs.

Fractionation of Secretions.

Nasal secretions from the lambs in each group, collected on days 11, 13 and 15 after inoculation, were pooled and fractionated by gel filtration. After concentration, the HI titres and immunoglobulin levels

Table 4.

Nasal Secretion HI Titres *

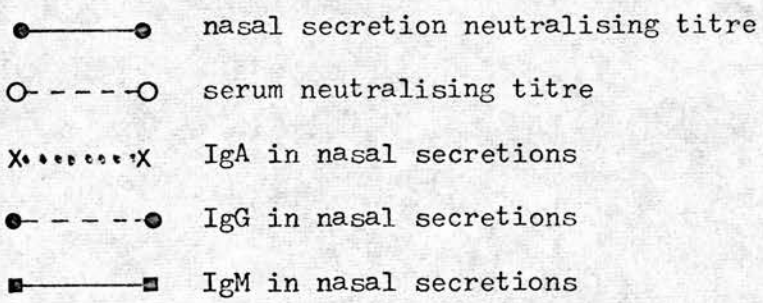
Days after Inoculation

Group	Lamb	0	3	5	7	9	18	** 23	26	29	32	36
Infected	866	8	8	8	16	16	8	8	8	8	8	8
	882	<8	<8	NT	<8	<8	8	8	8	8	8	8
	885	8	8	32	16	16	32	16	8	8	<8	16
	894	8	<8	<8	8	16	8	8	<8	8	8	8
Control	870	8	16	16	8	16	8	16	8	8	8	8
	886	8	<8	<8	<8	NT	<8	8	8	<8	8	8
	887	8	8	NT	<8	8	16	8	8	8	8	8

* after treatment with RDE

** samples collected on day 11, 13 and 15 were pooled for fractionation.

Fig. 17 Immunoglobulin concentrations and neutralising antibody titres in the nasal secretions of the infected lambs.



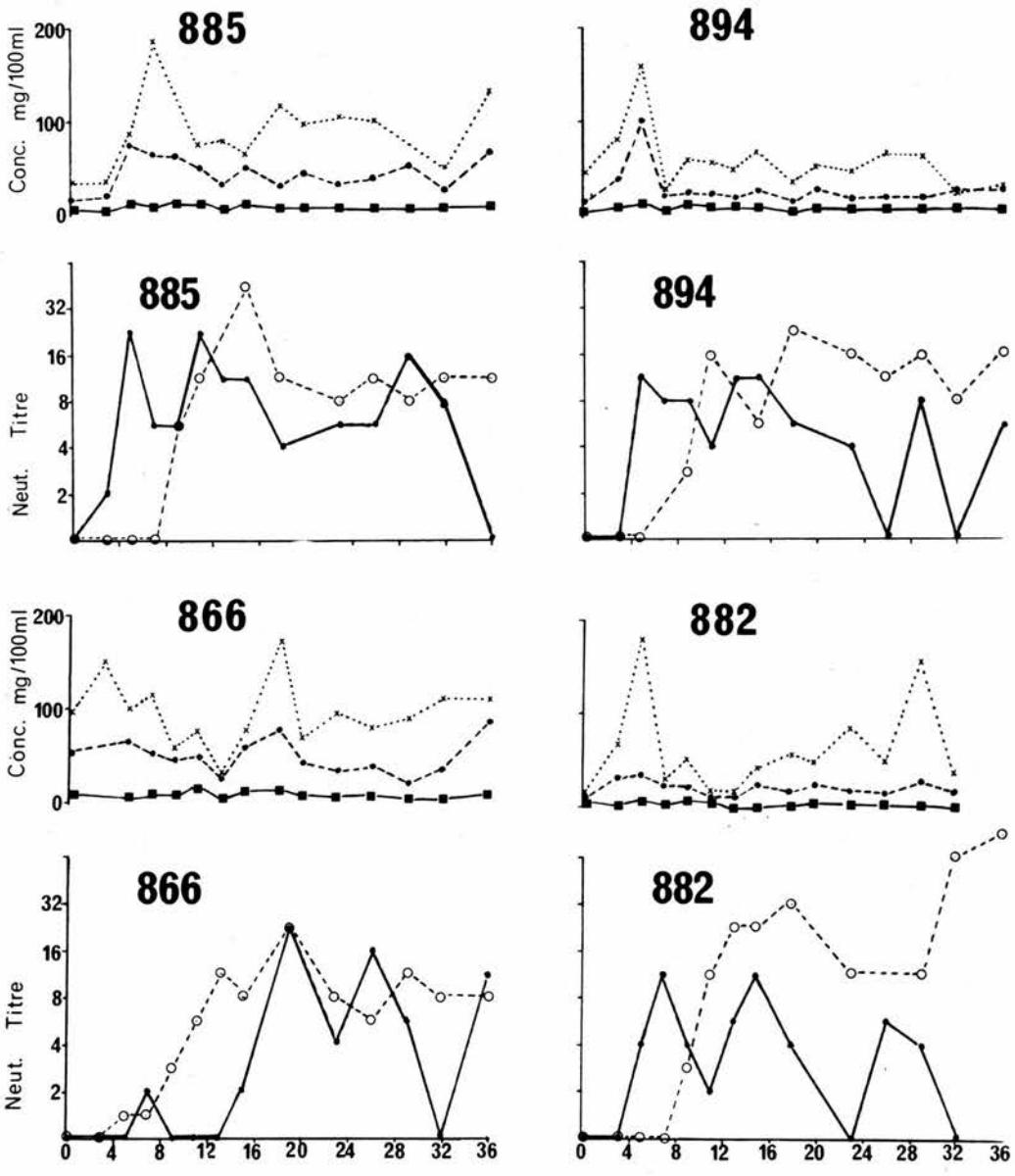
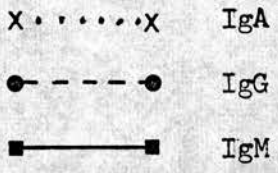
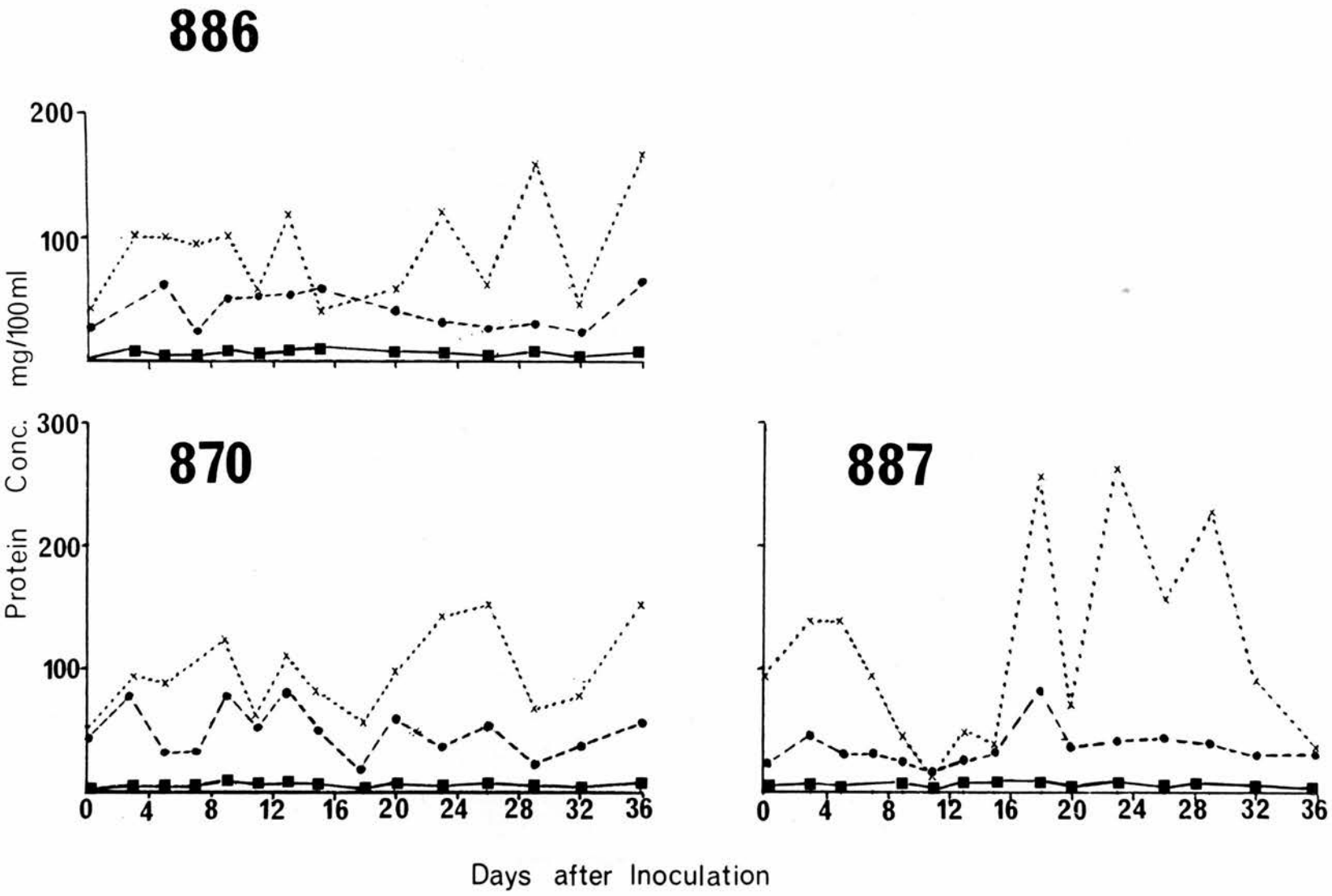

885
894
Conc. mg/100ml
200
100
0
885
894
Neut. Titre
32
16
8
4
2
866
882
Conc. mg/100ml
200
100
0
866
882
Neut. Titre
32
16
8
4
2
0 4 8 12 16 20 24 28 32 36
Days after Inoculation

Fig. 18 Immunoglobulin concentrations in the nasal secretions of the control lambs.




886
200
100
Protein Conc. mg/100ml
870
300
200
100
887
0 4 8 12 16 20 24 28 32 36
0 4 8 12 16 20 24 28 32 36
Days after Inoculation

of each fraction were assayed. It was found that each pool of secretions contained similar protein and immunoglobulin concentrations and that the HI activity in the secretions from the control group was eluted as a peak at the exclusion volume of the column (Fig. 19). HI activity was found in this area in the nasal secretions from the infected group, but activity was also present in the fractions containing IgA and no HI activity was detected in the fractions containing IgG. The experiment was repeated with a pool of secretions collected 18 to 36 days after inoculation and similar results were obtained.

Fractions from nasal secretions of the infected group were made into 3 pools termed A, B and C each containing only IgM, IgA and IgG respectively (Fig. 19). After concentration each pool was tested for HI activity to PI_1, PI_2, PI_3 and NDV. Pool A inhibited the HA of all 4 viruses, Pool B inhibited PI_3 only and no activity was found in Pool C (Table 5). In addition each pool was treated with RDE and the HI activity to PI_3 was retitrated. The titre of Pool B was not affected by RDE, whereas a four-fold drop in activity was found in Pool A (Table 6).

The post-inoculation tracheo-bronchial samples collected from the infected lambs were pooled and fractionated. Two peaks of neutralising activity were resolved (Fig. 20). The first was eluted in fractions near the exclusion volume; IgM was not detected in these or any other fractions. The second peak was eluted in association with IgA, although several of the later fractions in this peak also contained IgG. HI activity was detected in the fractions containing neutralising activity, although the HI activity was not resolved into 2 peaks so clearly. Substances with high absorbance at 254 nm were eluted after IgG. These fractions were not visibly coloured but tests showed that the absorbance could be markedly reduced by dialysis against PBS.

Fig. 19 Fractionation of nasal secretions from infected and control lambs by filtration through Biogel A 1.5 m.

Column: 70 x 2.5 cm. Fraction size: 10 ml.

Samples: 2 ml of nasal secretions pooled and concentrated from samples collected from each group between 11 and 15 days after inoculation.

After antibody titres and immunoglobulin levels had been estimated, fractions from the infected lambs were made into 3 pools, termed A,B and C, as depicted.

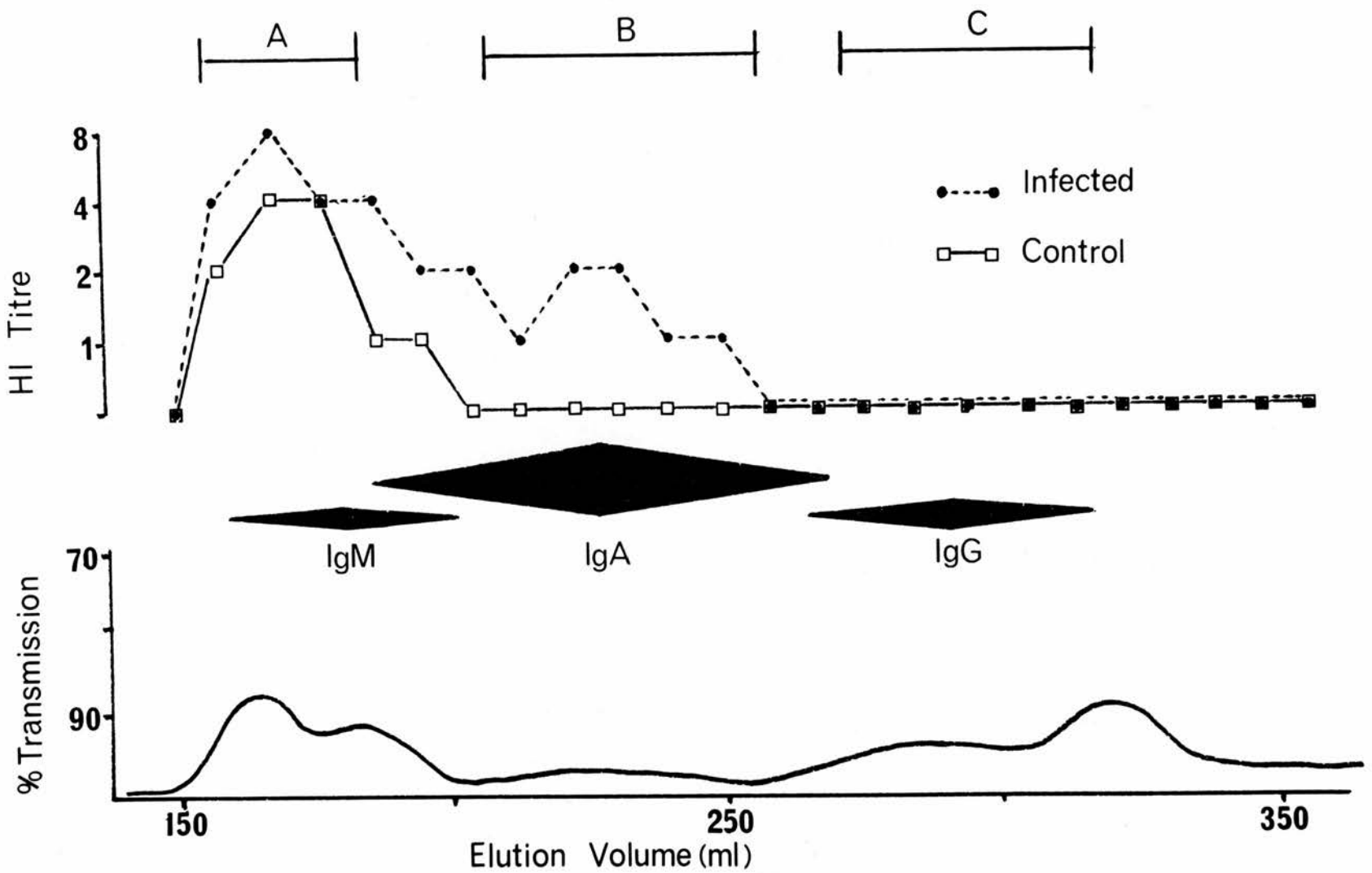

A
B
C
8
4
2
1
HI Titre
Infected
Control
IgM
IgA
IgG
70
90
% Transmission
150
250
350
Elution Volume (ml)

Table 5.

HI activity in 3 nasal secretion fractions to PI_3, PI_2, PI_1 and NDV viruses.

Virus	Fractions A	B	C
PI_3	80	40	<2
PI_2	8	<2	<2
NDV	8	<2	<2
PI_1 (Sendai)	8	<2	<2

Table 6.

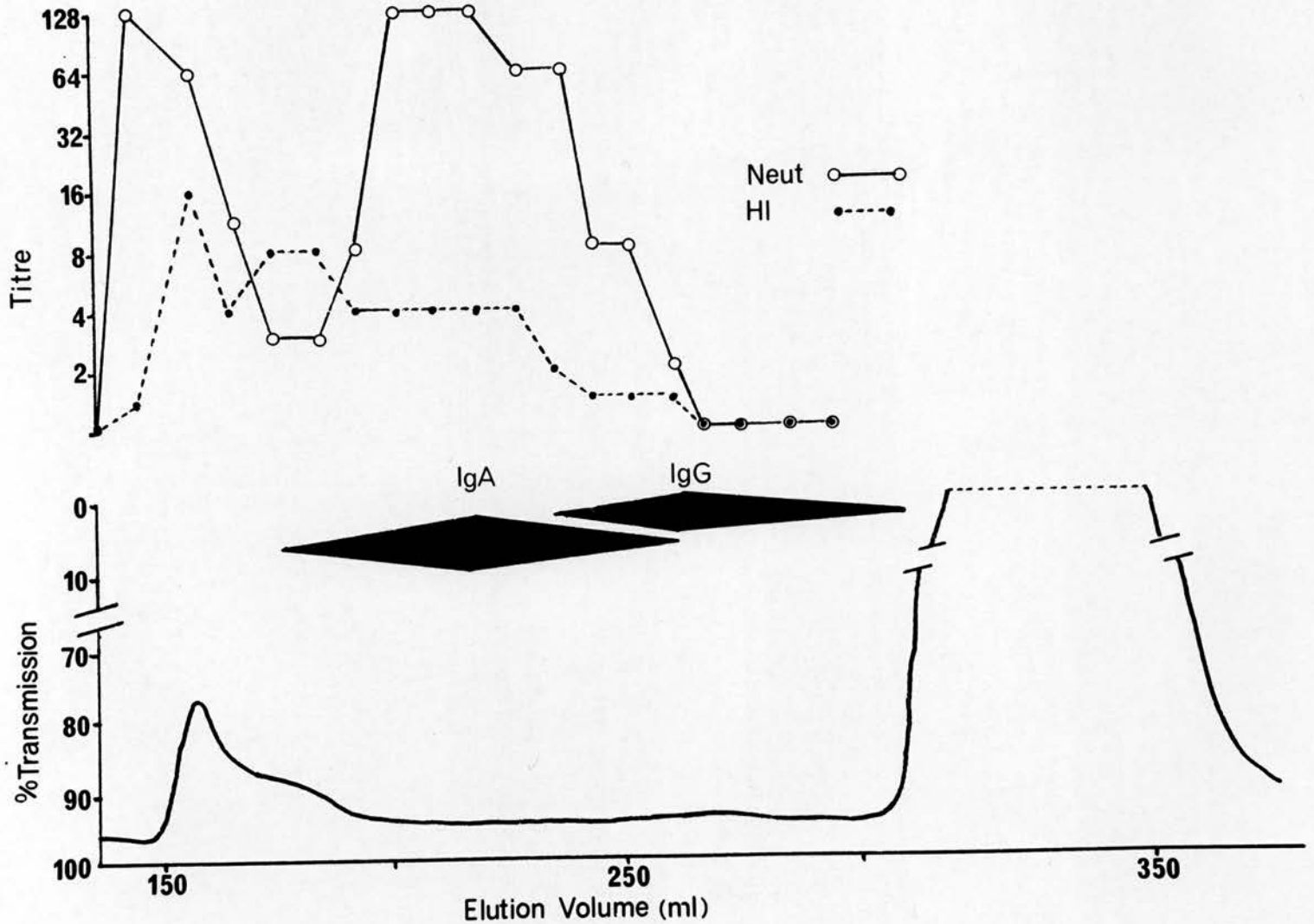
The effect of RDE on the HI activity of these fractions to PI_3.

Treatment*	Fractions A	B	C
RDE	20	40	<10
PBS	80	40	<10

* sample diluted 1/5 with RDE or PBS

Fig. 20 Fractionation of pooled tracheo-bronchial secretions from the infected lambs by filtration through Biogel A 1.5 m.

Column: 70x 2.5 cm. Fraction size: 10 ml. Sample: 3 ml.


128
64
32
16
8
4
2
Titre
Neut
HI
IgA
IgG
0
10
70
80
90
100
%Transmission
150
250
350
Elution Volume (ml)

Delayed hypersensitivity test. This was carried out 50 days after inoculation. At this time 2 of the lambs which had been inoculated with virus had serum HI titres greater than 10, whereas the remaining lambs were seronegative for PI_3. Before intradermal inoculation, the skin thickness of each lamb measured between 3 and 4 mm. No significant increase in thickness was detected at any inoculation site on any lamb when the skin was measured 24, 48 and 72 hours later.

Discussion

Inoculation of lambs with PI_3 did not result in clinical illness, a finding which has been reported previously (Hore, 1968; Gilmour et al, 1968). However isolation of the virus from nasal swabs taken from the inoculated group provided evidence of infection of these lambs.

Most of the PI_3 HI activity in the nasal secretions was considered due to a non-antibody inhibitor. This large molecular weight substance was found in pre-inoculation secretions and secretions from control lambs. It was partially sensitive to RDE and inhibited the HA of PI_1, PI_2 and NDV. However HI activity, specific for PI_3 and insensitive to RDE, was found in association with IgA in the nasal secretions of the infected lambs only. This activity was therefore attributed to IgA antibody. In individual nasal secretion samples IgA HI antibody could not be detected and was probably masked by the non-specific inhibitors, since no significant increase in HI titre was found after infection. Therefore unless a simple method is found to specifically remove this inhibitor, HI seems an unsuitable test for measuring ovine nasal secretion antibody. The neutralisation test seems more appropriate since rises in nasal secretion titre were found only in the infected lambs. However after concentration, neutralising activity was detected in the tracheo-bronchial secretions associated with a large molecular weight substance presumed similar to the nasal secretion inhibitor.

Since the tracheo-bronchial secretions contained no detectable IgM, it seemed unlikely that any of the anti-viral effect noted in either respiratory secretion was due to specific IgM antibody. However, the second peak of HI and neutralising activity in the tracheo-bronchial secretions was considered largely due to IgA antibody. Some IgG antibody may have been present in the tracheo-bronchial secretions but no HI activity was found in the IgG fraction of the nasal secretions.

Nasal secretion antibody was usually detected before serum antibody, which suggests that nasal antibody is independent from serum antibody. Similar findings have been reported in calves (Morein, 1972). It was not possible to associate the appearance of nasal antibody with changes in the nasal secretion immunoglobulin concentrations since marked day to day variations were found in both groups of lambs. Immunoglobulin levels in bovine nasal secretions have also been shown to vary considerably from week to week (Duncan et al, 1972).

In the serum, HI antibody was detected before neutralising antibody as noted by Hore (1968). Nasal secretion antibody did not persist as long as serum antibody. Similar findings have been reported in calves (Marshall and Frank, 1971), although in this species nasal antibody was detected more than 17 weeks after infection as compared with the 4 to 5 week period found here. However, this may reflect differences in the sensitivity of the neutralisation tests employed, or differences in the dose of virus administered, since Marshall and Frank (1971) also reported reduced nasal antibody titres in 2 calves infected with smaller doses of PI_3.

The results of the intradermal test were inconclusive since no positive control situation was available to test the efficacy of the skin test antigen. It is possible that the antigen was denatured in some way, although it did have a HA titre. Recent findings in the

bovine indicate skin hypersensitivity following natural infection with PI_3 (Morein and Moreno-Lopez, 1973).

PART 2

Antibodies and Immunoglobulins in the Serum and Nasal Secretions of Lambs following Vaccination and Aerosol Challenge with Parainfluenza 3 Virus

EXPERIMENT I

Introduction

In this experiment serum and nasal antibody titres were assayed in SPF lambs, inoculated with live or inactivated PI_3 given either IN or IM. Any protection afforded by either of these 4 types of vaccination was then assessed by exposing the animals to an aerosol of live virus. Serum and nasal secretion IgA, IgG and IgM levels were estimated in an attempt to discover which immunoglobulins were participating in the response.

Scheme of Experiment. Twenty-seven 10 day old Dorset SPF lambs were randomly placed in 5 groups of 4 and 1 group of 7 (Table 7). Group 1 was inoculated IN and Group 2 IM with fluid containing 10^6 TCID 50 PI_3/ml. Inactivated virus, prepared from an aliquot of the same fluid, was administered by similar routes to Groups 3 and 4 respectively. The inoculum volume was 2.0 ml per lamb given either as a single IM injection into the hind leg or as 1.0 ml instilled into each nostril. Groups 5 and 6 were not vaccinated. Twenty-five days after vaccination all lambs except those in Group 6 were challenged by exposure to an aerosol of live virus. It was estimated that each lamb inhaled approximately $10^{4.9}$ TCID 50. All lambs were swabbed daily for 1 week following vaccination and for 10 days after challenge, and during these periods clinical observations and rectal temperatures were noted daily. Blood and nasal secretion samples were collected at weekly intervals after vaccination. All lambs were killed at the end of the

Table 7. Scheme of Experiment.

Group No.	No. of Lambs.	Vaccine Route & Type.	Aerosol Challenge
1.	4	Live IN	+
2	4	Live IM	+
3	4	Inactivated IN	+
4	4	Inactivated IM	+
5	4	None	+
6	7	None	-

Table 8. Titre of virus recovered from the nasal swabs of Group 1 after IN inoculation of 10^6 $TCID_{50}$ PI 3.

	Days after inoculation.						
Lamb No.	1	2	3	4	5	6	7
25	0.66*	>3.0	4.0	3.75	>5.0	4.0	2.5
27	-	1.66	2.0	2.33	4.66	2.66	1.5
28	-	NT	-	0.66	1.66	1.66	2.3
29	0.33	1.8	2.1	2.5	4.33	2.0	1.66

* Log $TCID_{50}$/ 0.2 ml of transport medium

\- No virus recovered

NT Not tested

experiment and pieces of the respiratory tract were removed for histological examination.

Results

Virus recovery and antibody response

Response to Vaccination. No clinical sign or pyrexia was observed in any of the lambs except in Group 1 where a mild serous nasal discharge was noted during the period of virus excretion.

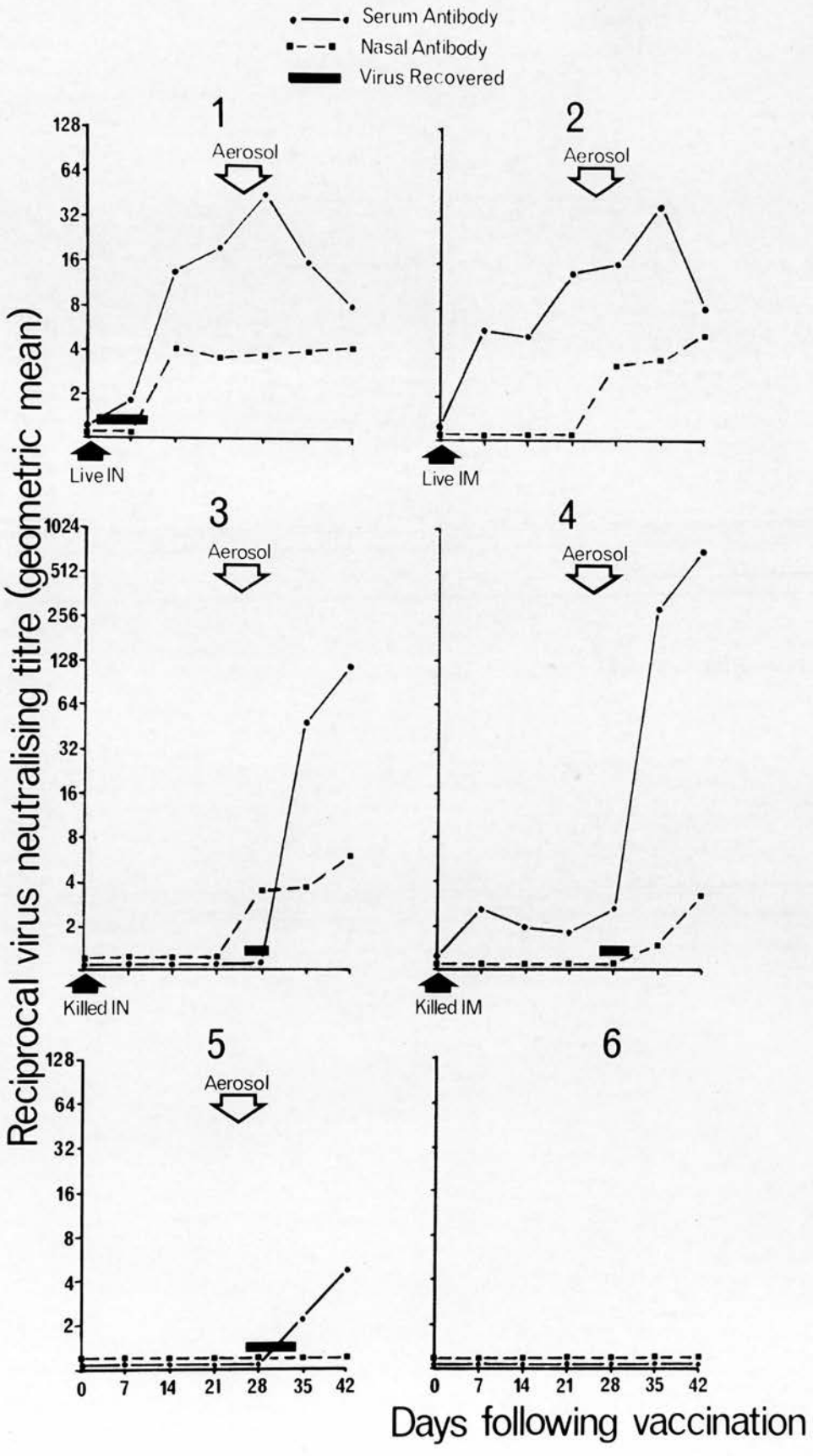
Virus was recovered from all lambs in Group 1 following IN inoculation with live virus and titration of the swabs showed that there was an increase in virus shedding from day 2 until day 5 (Table 8). Neutralising antibody was first detected in the serum 7 days post-infection at a group geometric mean titre of 1.8 and increased to a titre of 20 at 3 weeks (Fig. 21). Nasal antibody was detected in 3 lambs 14 days post infection at titres of 5, 6 and 11 and was present in all four lambs by day 21. In Group 2 no virus was isolated from nasal swabs during the first week following inoculation. However, a serum neutralising antibody response comparable to that of Group 1 occurred but no antibody was detected in the nasal secretions. Lambs in Group 3 and those in Group 5 and 6 had no serum or nasal antibody before challenge, while only low serum neutralising antibody titres were detected in three of the lambs in Group 4 (Fig. 21).

Response to Challenge with an Aerosol of Virus

A transient nasal discharge was the only clinically detectable response to challenge and this was observed only in those lambs shown to excrete virus (Table 9).

No virus was recovered in Group 1 and a 2-fold increase in serum neutralising antibody was evident 3 days after challenge. However, no concomitant increase was observed in the nasal secretion antibody levels

Fig. 21


Serum Antibody
Nasal Antibody
Virus Recovered
1
Aerosol
Live IN
2
Aerosol
Live IM
3
Aerosol
Killed IN
4
Aerosol
Killed IM
5
Aerosol
6
Reciprocal virus neutralising titre (geometric mean)
128
64
32
16
8
4
2
1024
512
256
0 7 14 21 28 35 42
Days following vaccination

and serum titres returned to prechallenge levels within 17 days. similarly lambs in Group 2 did not excrete virus following challenge and demonstrated little rise in serum antibody levels, but in this group, where vaccination had not induced a nasal secretory antibody response, neutralising antibody appeared in the nasal secretions by the third day following challenge and these titres remained at comparable levels to Group 1 until the end of the experiment.

Virus was recovered from all 4 lambs in Group 3 on at least 2 occasions in the post-challenge period (Table 9). A nasal antibody response was detected 3 days after challenge in this group and preceded the serum response, which rose rapidly to a mean of approximately 128 by 17 days. Three lambs in Group 4 excreted virus (Table 9) and serum antibody levels increased very markedly after challenge in all the animals in this group. Nasal antibody however was only detected in one animal at 10 days and 3 animals at 17 days after challenge.

The control lambs in Group 5 all excreted virus which was recovered on at least 4 occasions after challenge (Table 9). A serum antibody response similar to that following vaccination of Groups 1 and 2 was evident but nasal antibody was not detected by the 17th day post-challenge. No serum or nasal antibody was ever detected in any of Group 6 lambs, which acted as controls to indicate any possible cross-contamination with virus between groups.

Pathology Findings

No gross or histological abnormalities were found in the lungs of any lamb. No inclusion bodies were observed in sections stained with Pollack's trichrome. Submucosal accumulations of polymorphonuclear leucocytes were found in some sections of nasal mucosa obtained from both challenged and control lambs. These lesions were attributed to inflammatory changes associated with the trauma of nasal swabbing.

Table 9. Virus recovered from nasal swabs of Groups 3, 4 and 5 after challenge.

	Lamb No.	1	2	3	4	5	6	7	8	9	10
Group 3 Inactivated IN	20	-	-	-	-	+	+	-	-	-	-
	21	-	-	-	-	+	+	-	-	-	-
	22	-	-	-	+	+	+	+	-	-	-
	24	-	-	-	-	-	+	+	-	-	-
Group 4 Inactivated IM	5	-	-	+	+	+	-	-	-	-	-
	6	-	-	-	-	-	-	-	-	-	-
	8	-	-	-	+	+	-	-	-	-	-
	9	-	-	-	+	-	-	-	-	-	-
Group 5 Controls	11	-	-	+	-	+	+	+	-	-	-
	12	-	-	-	+	+	+	+	-	-	-
	13	-	-	+	-	+	+	+	+	-	-
	30	-	-	NT	+	+	+	+	+	+	-

\+ Virus recovered

\- No virus recovered

NT Not tested

Immunoglobulins Participating in the Response

Serum. Low levels of IgG and IgM were detected in the first serum samples collected 7 days after vaccination when the lambs were 17 days old. IgA was not found in these sera and was rarely detected in later samples (Fig. 22).

IgM was the predominant immunoglobulin in all sera on day 7 with individual levels ranging between 11 and 37 mg/100 ml in all the groups except No. 1 where higher levels (45 - 88 mg/100 ml) were recorded. Individual lamb IgG levels ranged from 2.7 to 20 mg/100 ml on this day. Mean (arithmetic) IgG and IgM concentrations in the control lambs (Group 6) rose at similar rates until day 28 when IgG increased more rapidly than IgM so that both immunoglobulins were at similar concentrations on days 35 and 42.

The IgM and IgG concentrations in Group 5 were equivalent to Group 6 until 3 days after challenge when IgM increased rapidly, reaching a peak at 120 mg/100 ml on day 35. This rise coincided with the appearance of serum antibody but no concomitant IgG increase, relative to the controls, was observed.

In Groups 3 and 4, IgM and IgG concentrations similar to the controls were detected until after challenge when sharp increases in IgG, corresponding to the secondary antibody response, were apparent.

Seven days after IN infection with virus, the mean serum IgM concentration (61 mg/100 ml) in Group 1 was higher than in the control groups. Somewhat higher concentrations of IgG were noted in 2 animals on this day, but by day 21 mean IgG concentrations were approximately twice that of the control groups. After challenge the concentration of IgG overtook that of IgM and IgG was usually the predominant serum immunoglobulin.

Fig. 22

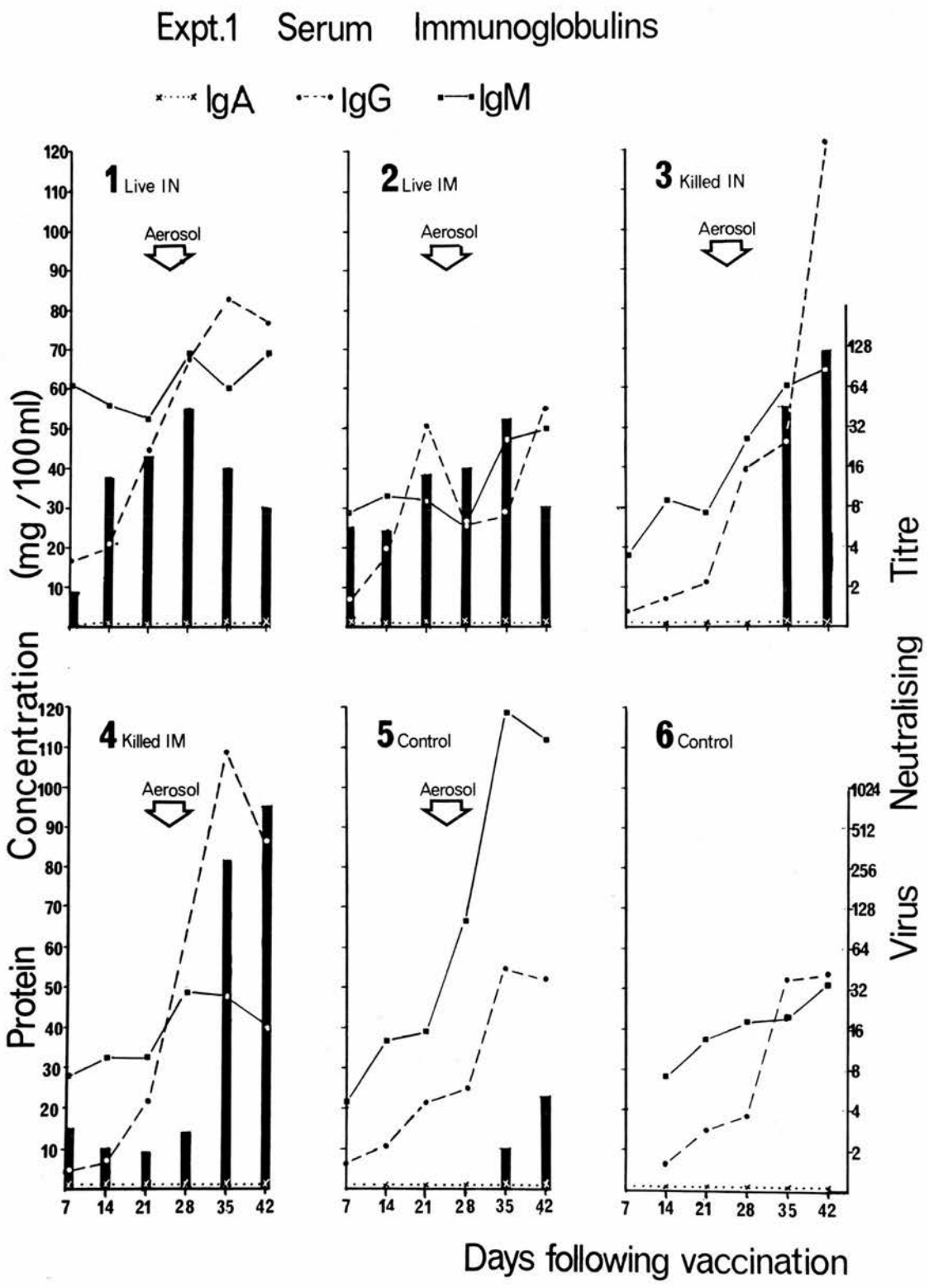

Expt.1 Serum Immunoglobulins
IgA
IgG
IgM
1 Live IN
2 Live IM
3 Killed IN
4 Killed IM
5 Control
6 Control
Aerosol
Protein Concentration (mg /100ml)
Virus Neutralising Titre
Days following vaccination

Fig. 23

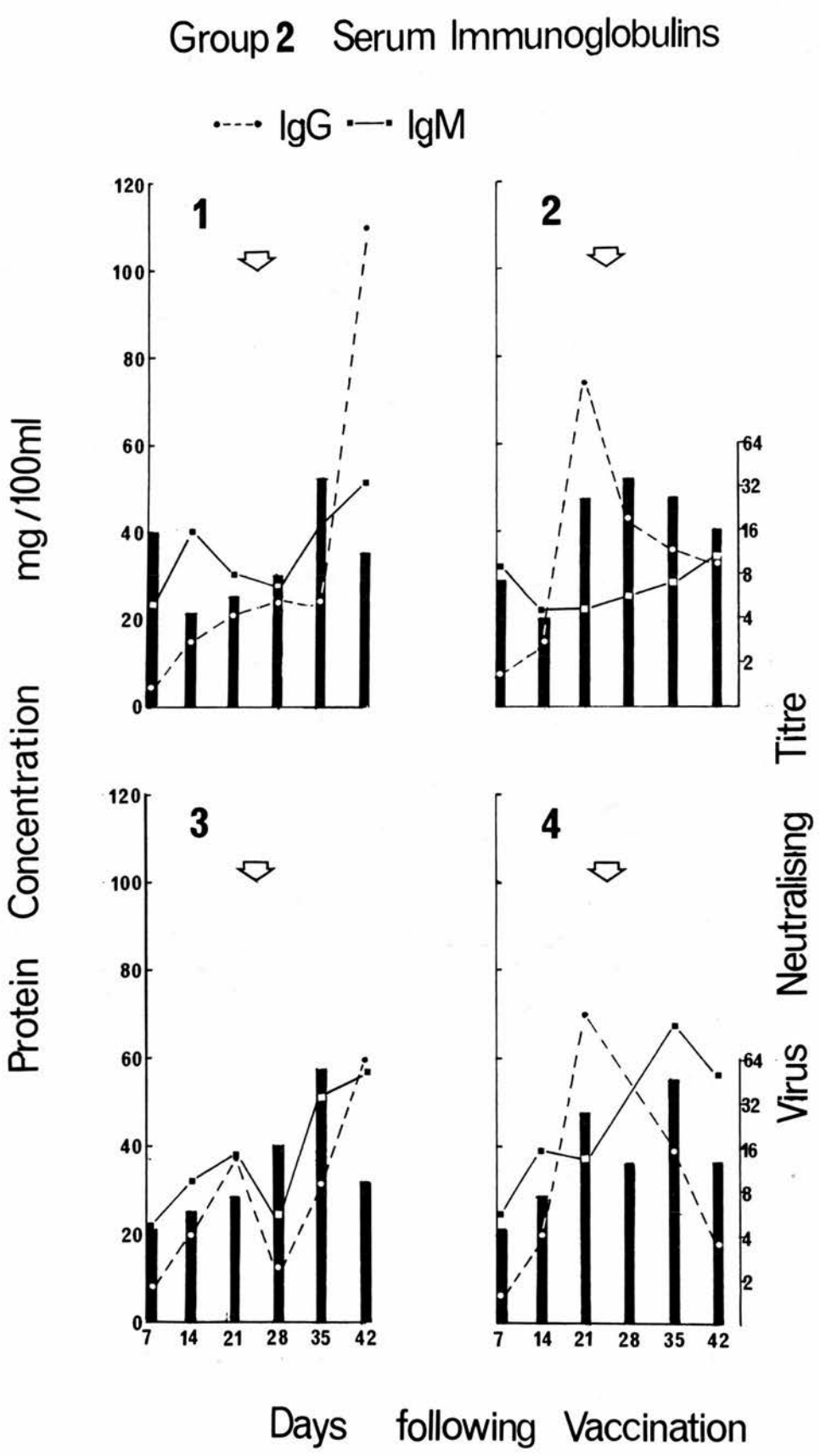
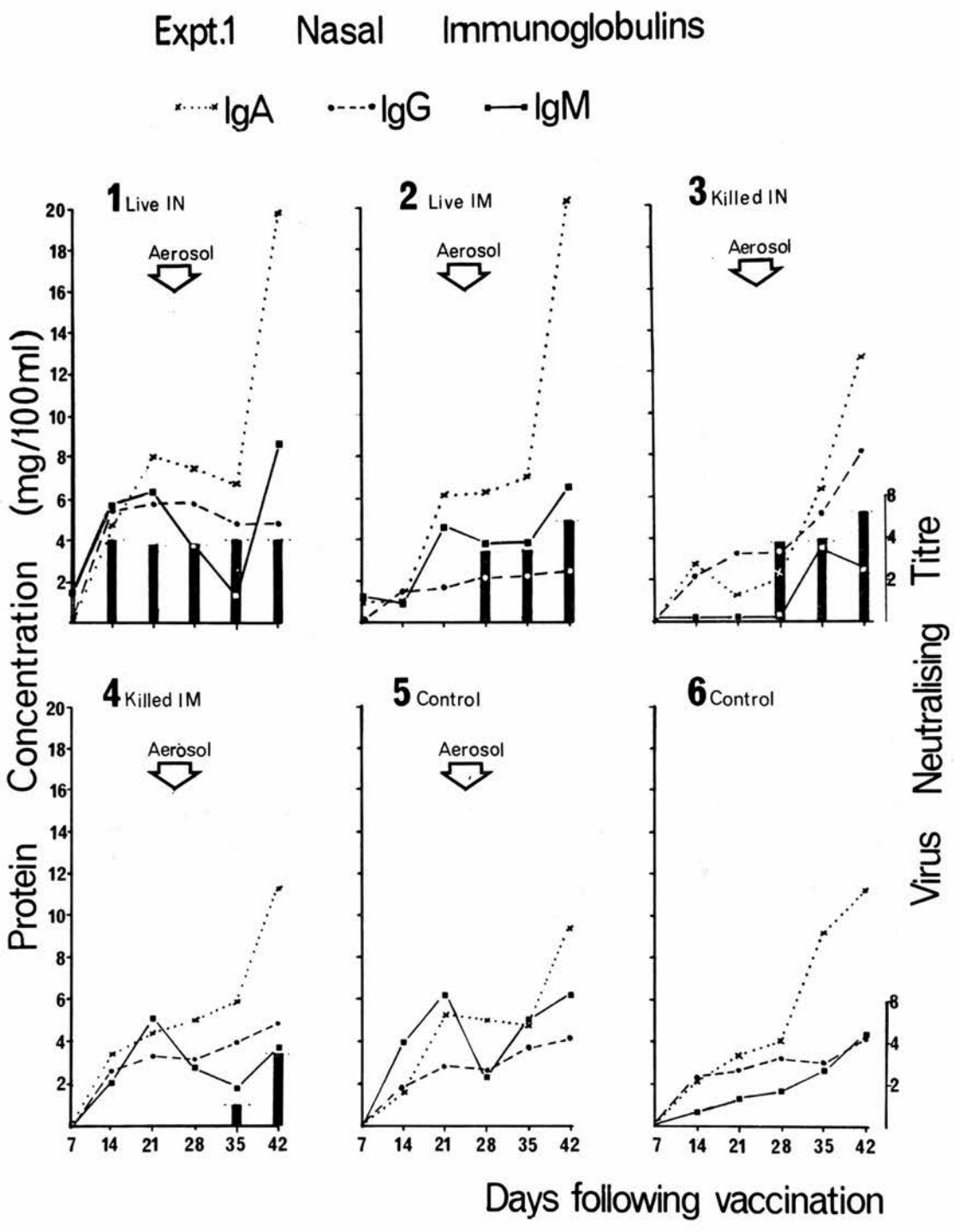

Group 2 Serum Immunoglobulins
IgG
IgM
1
2
3
4
Protein Concentration mg/100ml
Virus Neutralising Titre
Days following Vaccination
0
20
40
60
80
100
120
2
4
8
16
32
64
7
14
21
28
35
42

Fig. 24


Expt.1 Nasal Immunoglobulins
IgA
IgG
IgM
1 Live IN
2 Live IM
3 Killed IN
4 Killed IM
5 Control
6 Control
Aerosol
Protein Concentration (mg/100ml)
Virus Neutralising Titre
Days following vaccination

Individual lambs in Group 2 showed varying IgG and IgM responses (Fig. 23). Although no high initial IgM level was noted, in lambs 2 and 4 IgG increased sharply to a peak concentration on day 21. After challenge neither of these animals showed a secondary IgG rise although lamb 4 did show an increase in IgM levels. In Lamb 1 no rise in IgG occurred on day 21 but this immunoglobulin did increase sharply between days 35 and 42. Lamb 3 showed a response intermediate between these extremes with smaller increases of IgG on day 21 and after challenge.

Nasal Secretions.

Immunoglobulin was only detected in the occasional nasal secretion sample collected on day 7 (Fig. 24). IgA and IgG were detected in 4 of the control animals (Groups 5 and 6) on day 14 at between 2 and 4 mg/100 ml and IgM was found at similar concentrations in 4 of these lambs. In Group 6, IgA marginally predominated on days 21 and 28, becoming the major immunoglobulin constituent on days 35 and 42 with mean concentrations of approximately 8 and 11 mg/100 ml respectively (Fig. 24).

With the possible exception of Group 1, all the other groups showed a similar pattern, with IgA usually predominant especially on days 35 and 42. In Group 1 the mean concentrations of all 3 immunoglobulins were greater on days 14 and 21, which coincided with the detection of nasal antibody. However in the other groups no correlation between immunoglobulin concentration and nasal antibody was observed.

Discussion

The absence of clinical signs or pathology in SPF lambs following exposure to PI_3 either IN, IM or by aerosol emphasises the mildness of experimental infection with this virus. Thus virus excretion from the nose was the criterion used for assessing any protection conferred

by vaccination. Using this criterion, this experiment showed that live virus given either IN or IM was protective, whereas inactivated virus administered by either of these routes was not. Similar serum antibody levels followed inoculation of live virus either IN or IM. Lower levels were detected after IM administration of inactivated virus and no antibody was stimulated by inactivated virus IN.

HI activity was found in the control lambs' nasal secretions and therefore the HI test was not used to detect antibody. Nasal secretion neutralising antibody was detected only after IN vaccination with live virus. However after challenge, nasal antibody appeared more rapidly in Groups 2, 3 and 4 than in the control group (Group 5), suggesting that lambs may be capable of an anamnestic nasal antibody response. It is not clear why the lambs in Group 1 did not show such a response, but this may indicate that the immunity of this group was more solid and that these lambs were not infected. Responses, similar to the nasal antibody changes found in Groups 1 and 2, have been reported in calves (McKercher et al, 1972).

The IgA, IgM and IgG determinations made on Groups 5 and 6 provide information on the ontogeny of the immune response in the serum and nasal secretions of colostrum deprived lambs. At first IgM was the major serum immunoglobulin but within a few weeks IgG became predominant. No immunoglobulin was detected in the nasal secretions until the lambs were about 3 weeks old. During the next 2 to 3 weeks all three immunoglobulins were present in approximately equal concentrations, before IgA emerged as the predominant immunoglobulin class.

After Groups 3, 4 and 5 were challenged, IgM was clearly associated with the primary serum antibody response and IgG with the secondary response. The reason for the individual variation

in the Group 2 lambs' serum immunoglobulin response is obscure.

In the nasal secretions it was not possible to correlate changes in immunoglobulin concentrations with detection of antibody. This was probably due to the considerable variation in immunoglobulin levels found in individual nasal secretion samples, as described previously in conventional sheep (Section II part 2 and this section, part I). Unfortunately not enough fluid of sufficiently high antibody activity was available to permit fractionation of the nasal secretions. Therefore antibody and immunoglobulin class could not be associated by this technique.

EXPERIMENT 2

Introduction

A single inoculation of inactivated virus stimulated a poor antibody response in the lambs in Experiment 1. This second experiment was carried out to test the effect of 2 inoculations of inactivated virus, given either IN or IM, with the IM dose emulsified in FCA. The effect of giving the first vaccine IM followed by an IN 'booster' without adjuvant was also investigated. Any immunity conferred by vaccination was assessed as before by challenging the lambs with an aerosol of virus. Attempts were made to determine the immunoglobulins participating in the response.

Scheme of Experiment. Sixteen, 10 day old, cross-Suffolk SPF lambs were randomly placed into 4 equal sized groups numbered 7, 8, 9 and 10 (Table 10). Group 7 was vaccinated IM with 2 doses of inactivated virus emulsified in an equal volume of FCA. Group 8 lambs received 2 IN doses of the same vaccine, without adjuvant but diluted in an equal volume of PBS. The lambs in Group 9 were inoculated first IM with vaccine in FCA and then IN without adjuvant. The interval between vaccinations was 3 weeks. The lambs in Group 10 were not vaccinated. Prior to inactivation the titre of the vaccine was 10^8 TCID 50/ml. The volumes inoculated were 2.0 ml IM and 1.0 ml into each nostril. Six weeks after the first vaccination all the lambs were challenged, group by group, by exposure to an aerosol of live PI_3. It was estimated that each lamb inhaled $10^{5.3}$ TCID 50 of virus. All lambs were swabbed daily for ten days after challenge and during this period clinical observations and rectal temperatures were noted. Blood and nasal secretion samples were first taken prior to vaccination and at weekly intervals thereafter. The samples were assayed for neutralising antibody and immunoglobulin concentrations. A serum sample and

TABLE 10. Scheme of Experiment.

Group No.	1st Vaccination	2nd Vaccination	Aerosol Challenge
7	Inactivated IM + FCA	Inactivated IM + FCA	+
8	Inactivated IN	Inactivated IN	+
9	Inactivated IM + FCA	Inactivated IN	+
10	None	None	+

a pool of nasal secretions both collected from Group 9 lambs just before challenge were fractionated by gel filtration to determine the immunoglobulin classes associated with the anti-viral activity.

Results

Response to Vaccination

Following the vaccination of Group 7, serum antibody was first detected in all the lambs 14 days later at a mean titre of about 13 (Fig. 25). Serum antibody titres continued to increase after the second vaccination reaching a mean of about 200 on day 42. Meanwhile nasal antibody was first noted 7 days after the first vaccination of this group and these secretion titres continued to rise after the 'booster' to a mean of almost 8 prior to challenge.

Group 9 lambs responded as those in Group 7 after the first vaccination. Following the IN instillation of inactivated virus, serum and nasal antibody appeared to rise more rapidly than Group 7, but the nasal titres of both groups were at similar levels by the day of challenge.

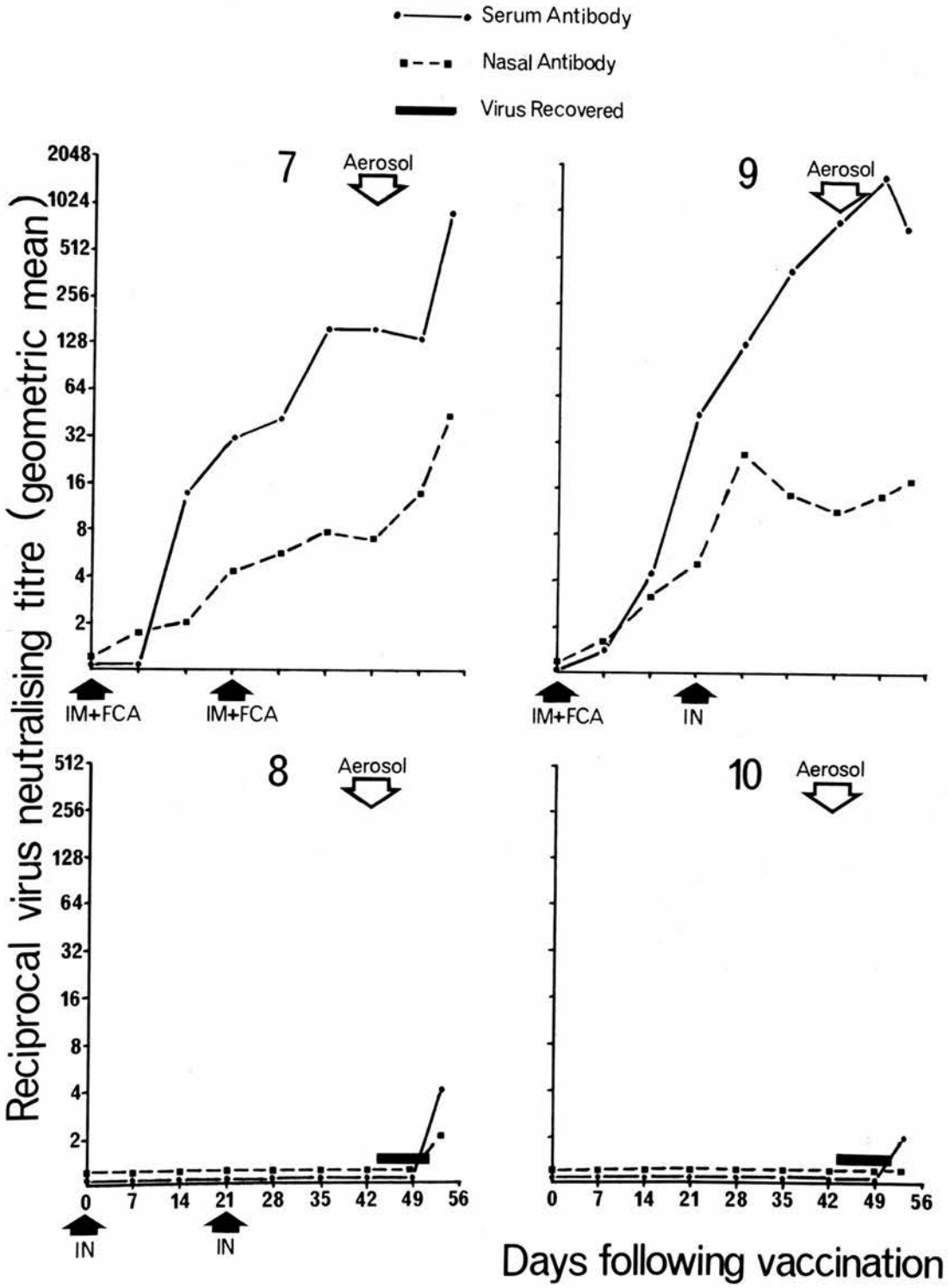
No detectable serum or nasal antibody response occurred in Group 8 following either vaccination.

Response to Challenge

Within approximately 2 hours of challenge all the lambs in Group 9 and 2 lambs in Group 7 were showing varying degrees of dullness, pyrexia and dyspnoea, and had rales and rhonchi. These signs gradually disappeared and within 3 days all lambs appeared clinically normal, except one in Group 9, which still had a respiratory lift.

These signs resembled an immediate-type hypersensitivity reaction which may have been caused by the animals being exposed to an aerosol of antigen to which they had previously been sensitised. As in the first experiment, the control (Group 10)

Fig. 25


Serum Antibody
Nasal Antibody
Virus Recovered
Reciprocal virus neutralising titre (geometric mean)
2048
1024
512
256
128
64
32
16
8
4
2
7
Aerosol
IM+FCA
IM+FCA
9
Aerosol
IM+FCA
IN
8
Aerosol
IN
IN
10
Aerosol
0 7 14 21 28 35 42 49 56
Days following vaccination

and IN vaccinated lambs (Group 8) were clinically normal following challenge, except for some nasal discharge. Similar amounts of virus were recovered from the lambs in these two groups (Table 11). Virus was never recovered from Groups 7 or 9.

By 12 days after challenge the mean nasal antibody levels in Group 7 increased approximately 4 fold, whereas little rise in titre was observed in Group 9. However, the mean serum antibody level in Group 7 fell slightly on day 49 but rose to about 1000 by day 54. In Group 9, on the other hand, mean serum titres increased to greater than 1024 on day 49 and fell 2 fold by day 54.

Twelve days after aerosol exposure all the Group 8 lambs had serum titres of 4 but only one lamb had detectable nasal antibody. Low serum antibody titres appeared in the control lambs' (Group 10) sera 12 days after challenge, but nasal antibody was not detectable at this time.

An attempt was made to discover the antigen(s) likely to have caused the hypersensitivity reaction in Groups 7 and 9. Gel diffusion tests showed that sera from lambs in these 2 groups had precipitating antibodies to horse serum, but not to any other constituents of the challenge inoculum or to lamb serum. No precipitins to horse serum were detected in the nasal secretions of the lambs. However, it was thought likely that the anaphylactic type reaction was due to horse serum antigens. Thus on day 52 of the experiment, 2 lambs in Group 9 were rechallenged with an aerosol of PI_3 grown up with medium containing 2% lamb serum and 2 lambs were rechallenged with identical medium, but without virus, and containing 2% horse serum.

Within an hour of challenge both pairs of lambs appeared dull and showed similar signs to those following the first challenge.

Table 11. Titre of Virus recovered from nasal swabs from lambs in Groups 8 and 10 after challenge.

		Days After Challenge								
	Lamb No.	1	2	3	4	5	6	7	8	9
Group 8 I.N. + I.N.	10	-	-	-	1.5*	1.0	0.7	0.5	-	-
	11	-	0.25	0.25	3.0	3.0	3.3	0.5	0.5	-
	13	-	0.15	1.7	2.3	1.0	3.3	0.15	-	-
	14	-	-	0.25	2.0	2.0	1.3	0.15	0.25	-
Group 10 Controls	12	-	0.15	0.5	2.5	0.7	1.3	0.15	0.15	-
	15	0.15	-	0.25	1.0	1.5	0.3	-	0.15	-
	16	-	0.15	0.3	1.0	2.5	1.3	0.25	-	-
	17	-	-	0.5	1.5	1.0	1.5	-	-	-

* Log $TCID_{50}$/.2 ml transport medium.

In addition, each of the pair exposed to virus had a very marked frothy nasal discharge, a sign not shown by the lambs exposed to horse serum. As was the case after the initial challenge, abnormal clinical signs had disappeared by 2 days, except for the same lamb as before, which still had a respiratory lift.

Immunoglobulins Participating in the Response

Serum

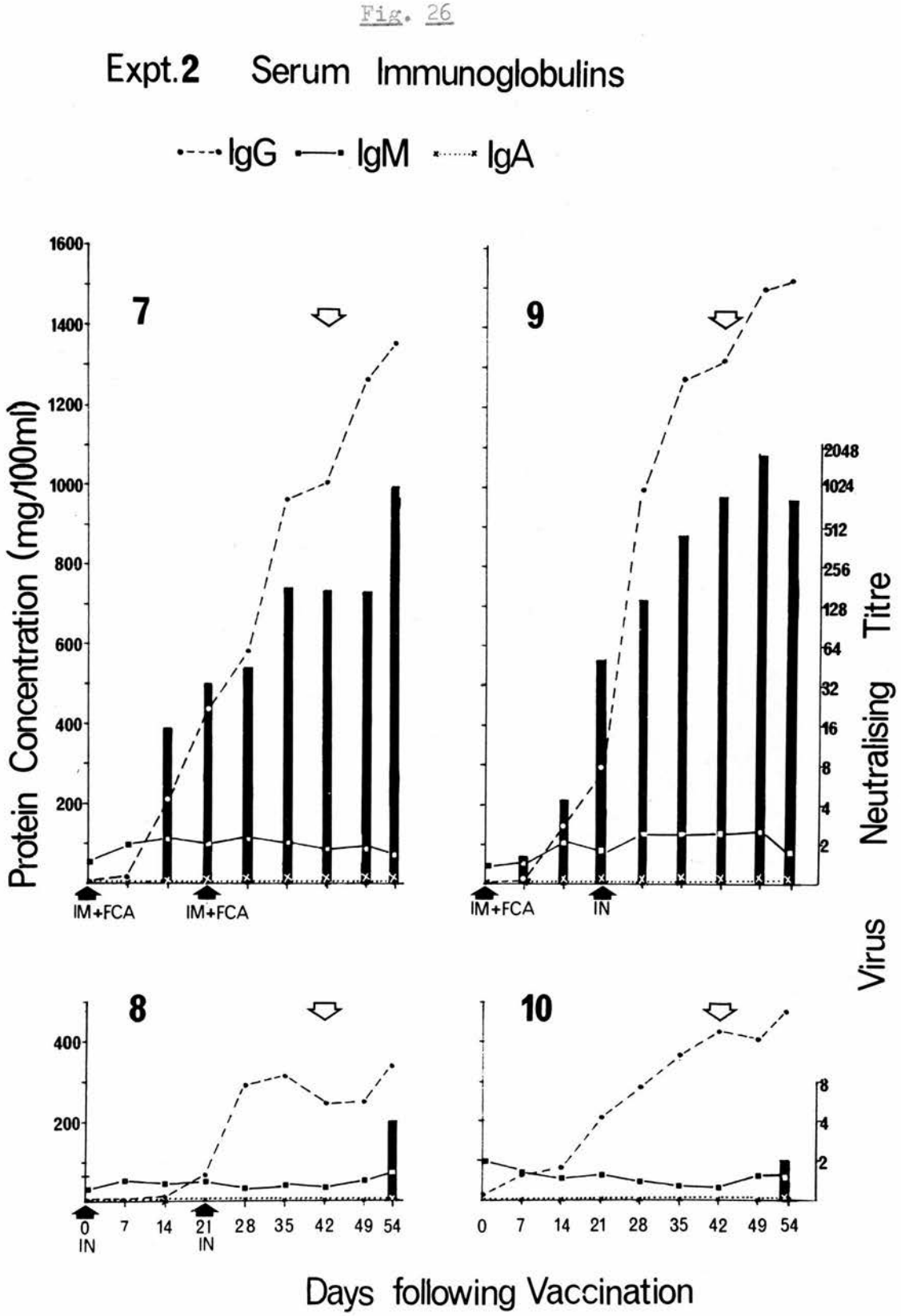
In the control lambs (Group 10) IgM was the major serum immunoglobulin at the beginning of the experiment with a mean concentration of 100 mg/100 ml (Fig. 26), but this decreased progressively until 7 days after the aerosol challenge when it increased from 35 mg/100 ml to 60 mg/100 ml. Mean IgG concentrations increased quickly from 20 mg/100 ml at day 0 to exceed IgM by day 14, reaching 400 mg/100 ml by day 42.

An essentially similar pattern was observed in Group 8 except that the mean initial IgM concentration was lower (30 mg/100 ml) and the increase in serum IgG occurred 1 week later. Like Group 10, both IgM and IgG increased after the challenge exposure and IgA was only occasionally detected at very low concentrations in the sera.

Seven days after vaccination with FCA, Group 7 lambs had higher serum IgM concentrations than the controls, increasing to 115 mg/100 ml by day 14 and thereafter fluctuating between 80 and 110 mg/100 ml. From day 7 onwards IgG increased much more rapidly than in the controls and this increase continued throughout the experiment reaching a mean of 1,350 mg/100 ml by day 54.

Serum immunoglobulin concentrations were very similar in Group 7 and 9 following vaccination with adjuvant. After the IN 'booster' however the serum IgG concentration in Group 9

Fig. 26


Expt.2 Serum Immunoglobulins
IgG
IgM
IgA
Protein Concentration (mg/100ml)
Virus Neutralising Titre
7
8
9
10
IM+FCA
IM+FCA
IM+FCA
IN
IN
IN
0
7
14
21
28
35
42
49
54
Days following Vaccination

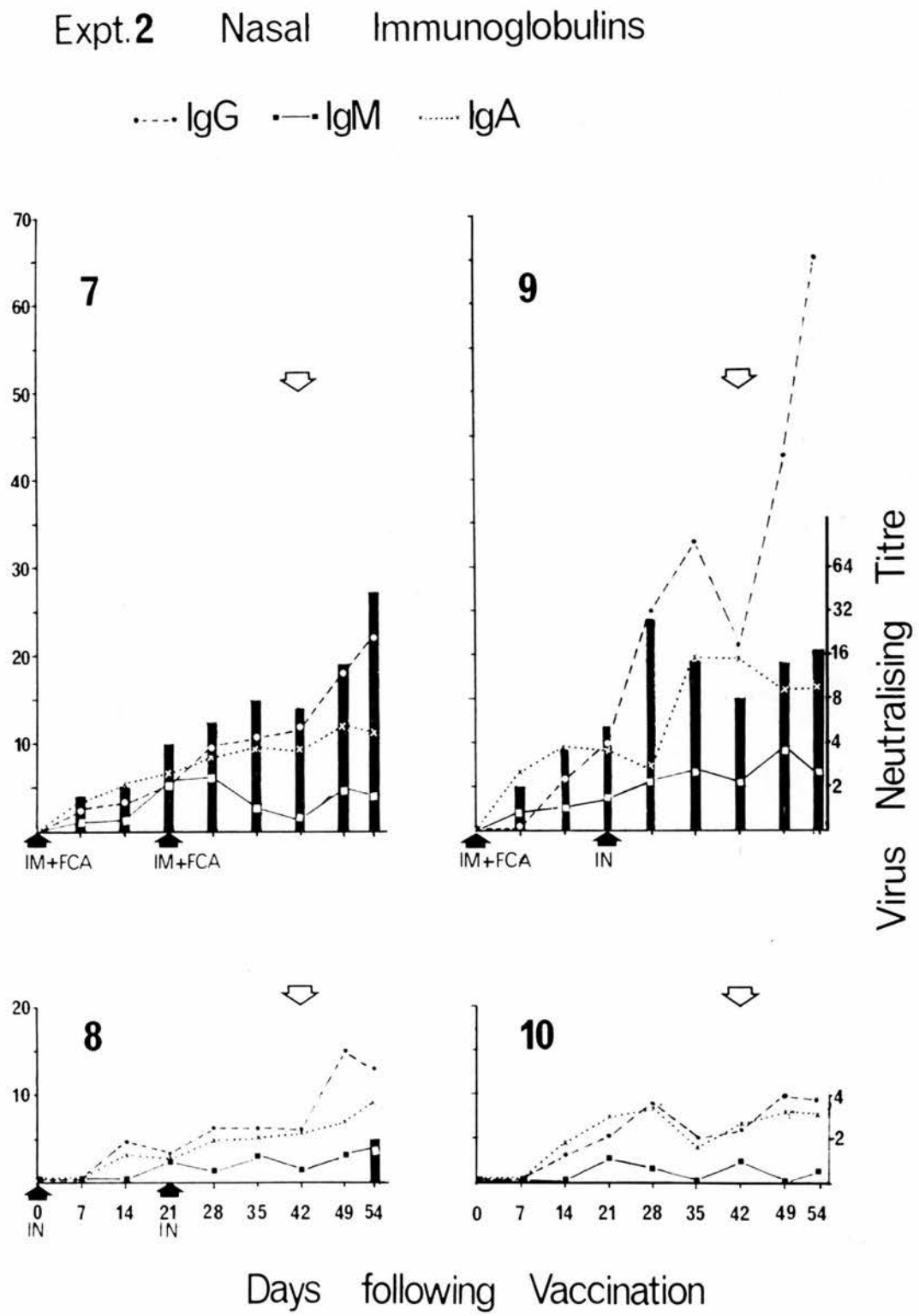
increased more rapidly than Group 7 to reach some 1000 mg/100 ml by day 28. Thereafter IgG in both groups increased at approximately similar rates so that Group 9 IgG concentrations were always some 200-300 mg/100 ml greater. Despite the high concentration of IgG and IgM, IgA was usually not detected in the sera of Groups 7 and 9.

Nasal Secretions

Immunoglobulins were not detected in any of the first nasal secretions samples collected. In the control lambs (Group 10) IgA and IgG were first detected on day 14 and similar mean concentrations of these immunoglobulins, ranging between 5 and 10 mg/100 ml, were detected throughout the experiment (Fig. 27). IgM was detected in most nasal secretions taken after day 21 at mean concentrations less than 5 mg/100 ml. The pattern observed in Group 8 was very similar except that higher concentrations of IgG were detected after the aerosol challenge.

Following the administration of adjuvant vaccine to Group 7 and 9, all 3 immunoglobulins were detected at higher mean concentrations than in the control lambs. After the second vaccination IgA and IgG concentrations in Group 7 continued to increase at similar rates each reaching some 10 mg/100 ml by day 42, whereas IgM concentrations decreased to a mean of 2 mg/100 ml over this period. However, by 1 week after the IN booster, a steep increase in IgG was observed in Group 9 continuing to day 35 when the mean IgG concentration was estimated at 32 mg/100 ml. An increase in nasal secretion IgA was also found in these lambs after the booster but this occurred 1 week after the IgG rise. IgM also increased during this period but like the IgG response a decline was noted between days 35 and 42. Following the aerosol challenge, increases in IgG and IgM were observed in Group 7 and 9 although the IgG response was much

Fig. 27


Expt. 2 Nasal Immunoglobulins
IgG
IgM
IgA
7
9
8
10
IM+FCA
IM+FCA
IM+FCA
IN
IN
IN
Virus Neutralising Titre
Days following Vaccination

greater in Group 9.

Fractionation of serum and nasal secretions.

A prechallenge serum sample from a lamb in Group 9 was filtered through the column of Biogel (Fig. 28). Neutralising and HI titres correlated closely and the antibody activity was confined to the 2 protein peaks which contained IgM and IgG, the bulk of activity being associated with IgG; IgA was not detected in any fraction.

A pool of prechallenge nasal secretions from Group 9 lambs was fractionated in an identical manner. Two peaks of anti-viral activity were resolved, corresponding to those observed with serum (Fig. 29). After 8-fold concentration, each fraction was tested for immunoglobulin content and re-titrated for anti-viral activity. Even after concentration no antibody was detected in the fractions containing IgA.

Samples of the serum and the pool of nasal secretions were analysed on immunoelectrophoresis with anti-IgG serum (Fig. 30). IgG_1 and IgG_2 precipitin arcs were resolved in the serum samples, but only IgG_1 was found in the nasal secretions.

Pathology

Post mortem findings. All lambs which had been inoculated with adjuvant had fibrous lesions and small beads of pus at the injection site. The lungs of the animals in Group 9 were rather heavy and oedematous. The lungs of lamb No. 6, which was clinically ill at slaughter were also consolidated along the costal margins of the diaphragmatic lobes and excess frothy material was present in the larger airways. No gross abnormalities were detected in the remaining lambs' lungs.

Histological findings.

Lung. No abnormal histological features were seen in the lung sections made from Groups 9 and 10, although erythrocytes were often present in

Fig. 28 Fractionation of serum by filtration through Biogel A 1.5 m.

Sample: 2 ml of serum collected from lamb No. 5 in Group 9 on day 42.

Column: 70 x 2.5 cm. Fraction size: 8 ml.

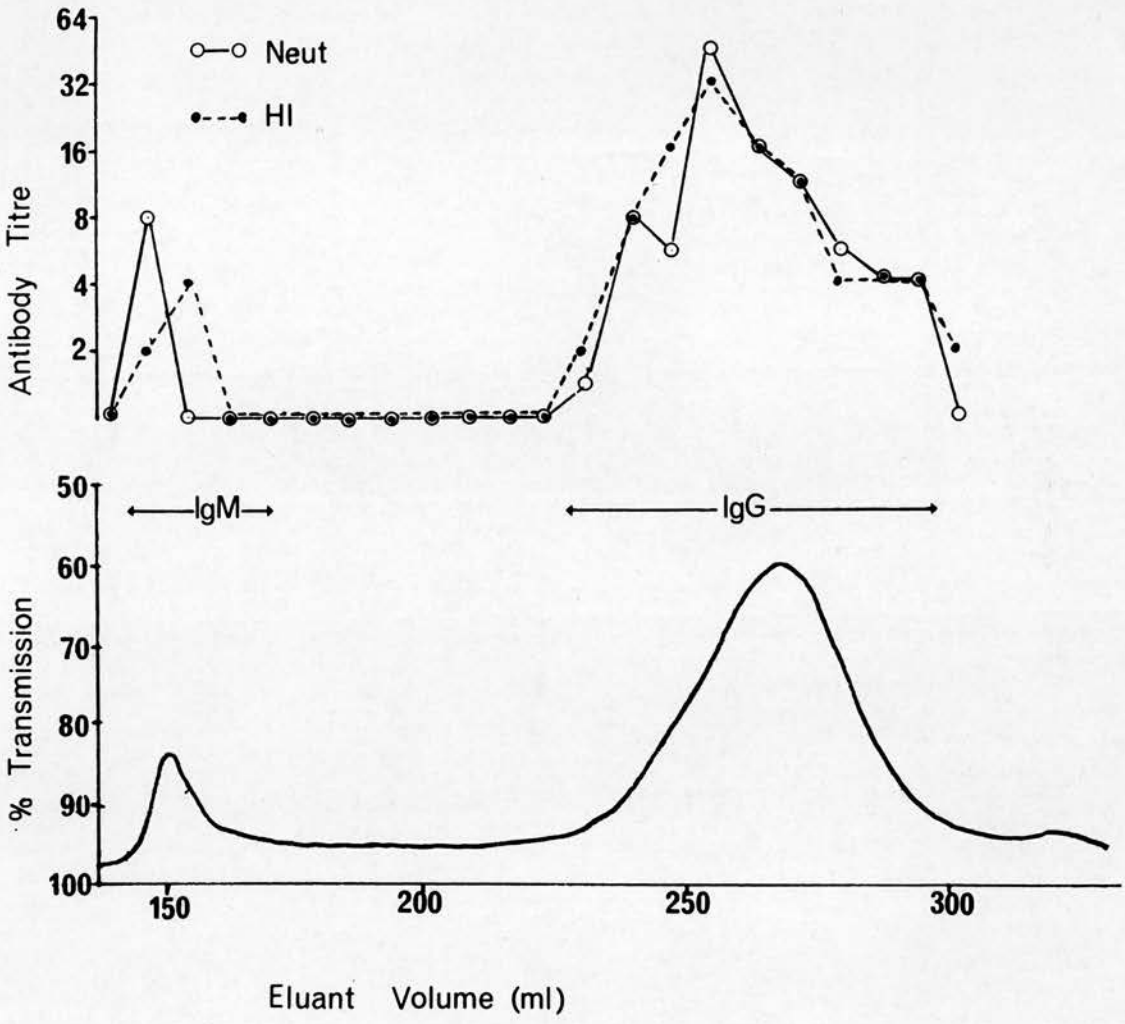
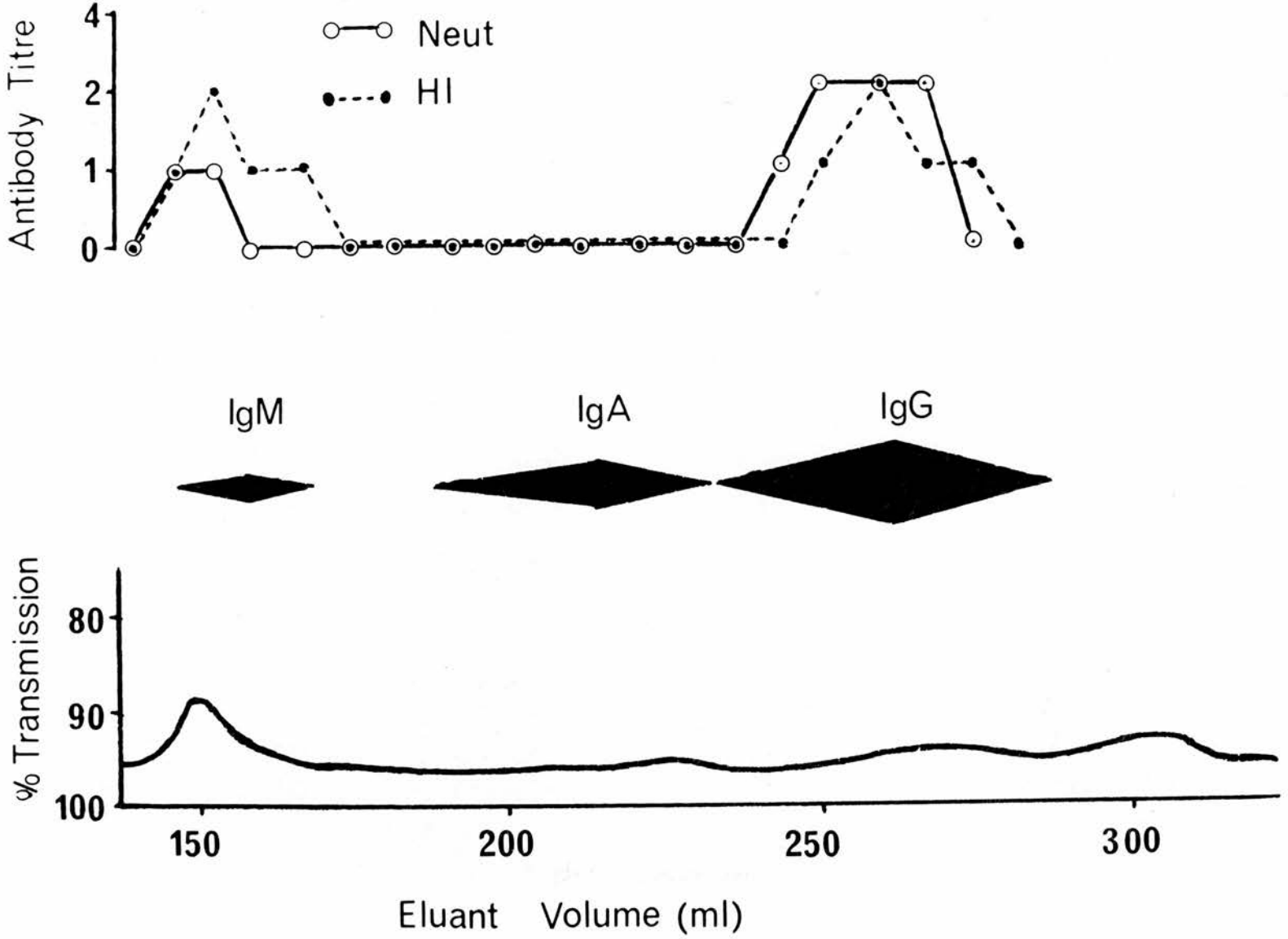

64
32
16
8
4
2
Antibody Titre
Neut
HI
50
60
70
80
90
100
% Transmission
IgM
IgG
150
200
250
300
Eluant Volume (ml)

Fig. 29 Fractionation of nasal secretions by filtration through Biogel A 1.5 m.

Sample: 3 ml of pooled nasal secretions collected from Group 9 lambs on day 42.

Column: 70 x 2.5 cm. Fraction size 8 ml.


Antibody Titre
4
2
1
0
Neut
HI
IgM
IgA
IgG
% Transmission
80
90
100
150
200
250
300
Eluant Volume (ml)

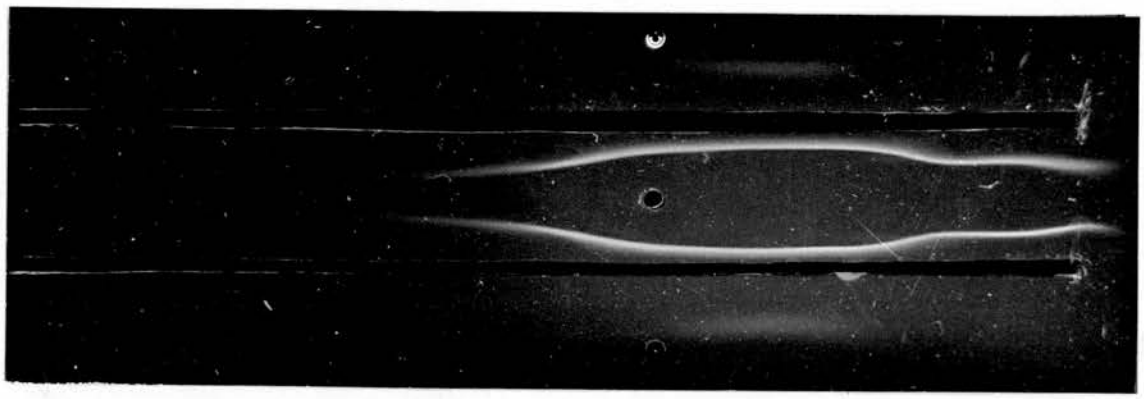


Fig. 30 Immunoelectrophoresis of serum and nasal secretions obtained from Group 9 on day 42.

upper and lower wells; nasal secretions.

centre well; serum.

troughs; anti-IgG.

The anode is on the left.

the airways due to inhalation of blood at slaughter (Fig. 31). No inclusion bodies were detected even in sections stained with Pollack's trichrome. Mild perivascular and peribronchiolar 'cuffing' caused by accumulation of lymphoid and mononuclear cells was the only abnormality detected in the lung sections of Group 7 (Fig. 32). This feature was also found in Group 9 lambs in addition to patches of interstitial pneumonia caused by invasion of lymphoid and mononuclear cells into the alveolar septa (Figs. 33 and 34). These lesions tended to be distributed near bronchioles but polymorphonuclear leucocytes were common throughout much of the tissue, often within the lumen of the bronchioles. These features were found to a similar degree in both of the pairs of lambs in this group.

Nasal mucosa, trachea and bronchus. Focal submucosal accumulations of polymorphonuclear leucocytes were usually observed in sections of the nasal septa taken from all groups of lambs. These were probably due to an inflammatory reaction following the trauma of nasal swabbing. Compared with the other groups, increased numbers of eosinophils and polymorphonuclear lymphocytes were seen in the nasal, tracheal and bronchial submucosae of the Group 9 lambs.

Discussion

In contrast to the results of Experiment 1, it was found that inactivated virus given with FCA by the IM route stimulated high antibody titres in both the serum and nasal secretions and these titres increased to very high levels after a second vaccination, either IM with FCA or IN without adjuvant. Nasal antibody titres were greater, albeit temporarily, when the second inoculation was given IN. However 2 IN instillations of inactivated virus did not stimulate any detectable antibody and these lambs and the control group shed similar quantities of

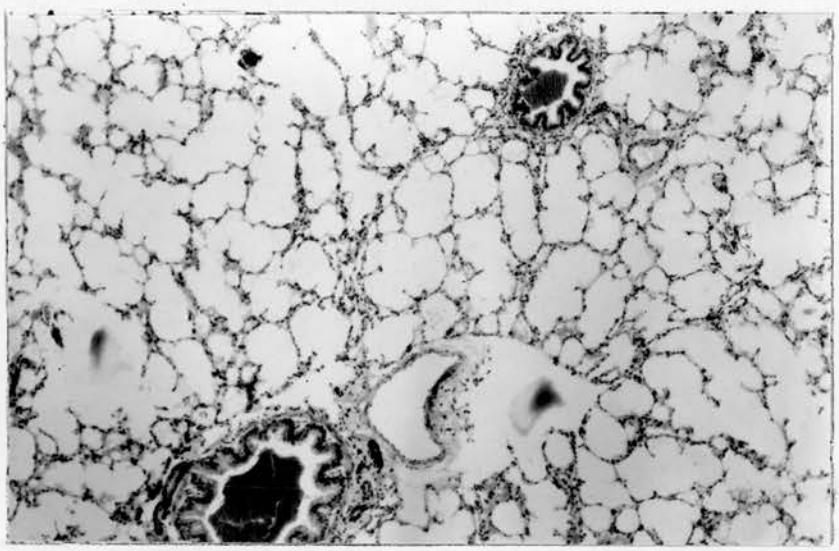


Fig. 31 Normal lung from lamb No. 13 in Group 10 (magnification x 60). Erythrocytes are present in the bronchioles due to backbleeding at slaughter.

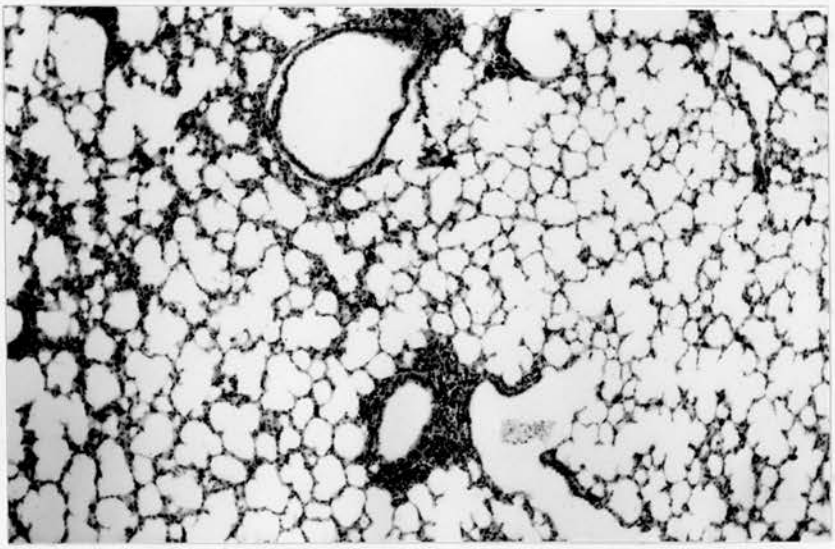


Fig. 32 Lung from lamb No. 4 in Group 7 (magnification x 60). A mild peribronchiolar accumulation of lymphoid and mononuclear cells can be seen.

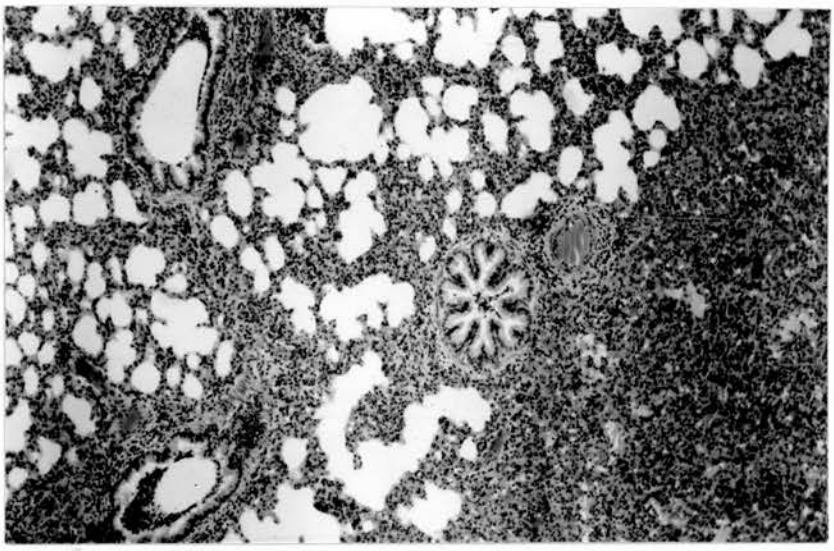


Fig. 33 Lung from lamb No. 8 in Group 9 (magnification x 60). This animal was rechallenged with PI_3 in serum-free medium. This section shows interstitial pneumonia caused by invasion of lymphoid and mononuclear cells into the alveolar septa.

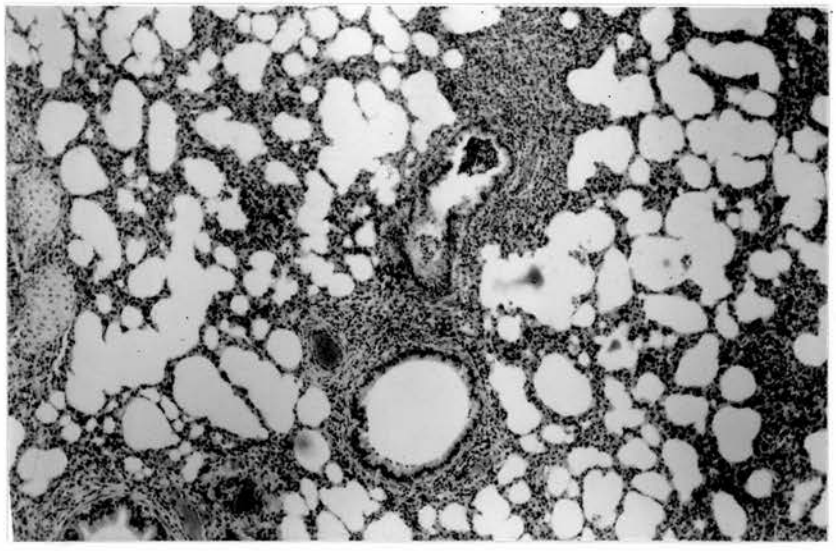


Fig. 34 Lung from lamb No. 6 in Group 9 (magnification x 60). This lamb was rechallenged with horse serum. The section shows interstitial pneumonia distributed mainly near bronchioles. Polymorphonuclear lymphocytes are present in the lumen of one bronchiole.

virus after challenge. Again, despite replication of the virus, abnormal clinical signs were not observed in the control lambs after challenge. No viral inclusion bodies or pathological changes were observed in histological sections taken from these animals' lungs. This was not surprising since Stevenson (1968) could only detect inclusion bodies up to 8 days after infection.

However the hypersensitivity reactions and lesions shown by the lambs in Groups 7 and 9 after challenge were unexpected. On the other hand FCA alone has been found to produce mild peribronchiolar 'cuffing' in calves (Wells, 1972) and anaphylaxis has been elicited in sheep, immunised with antigens in FCA (Alexander et al, 1970; Ladiges et al, 1974). In addition, experimental anaphylaxis with similar histological changes has been produced in sensitised cattle exposed to aerosols of the sensitising antigen (Dungworth, 1965). Similar cellular reactions to those seen in Group 9 lambs have been described by Bartlema and Fontages (1973, citing Karelse, 1970) in respiratory immunisation studies with mice. Following intratracheal vaccination with tetanus toxoid with a Bordetella pertussis adjuvant, Karelse found interstitial pneumonia with perivascular infiltration of cells. This cellular infiltration initially consisted of leucocytes but in later stages was predominantly mononuclear cells. Unfortunately it was not clear whether viral antigens were involved in the reactions seen in the experiment here. Obviously it would be of considerable importance to know if such a reaction could occur in naturally exposed lambs which had been immunised by this method.

It is not clear why serum immunoglobulin concentrations were considerably greater in the control animals in this experiment compared with Experiment 1. Although these lambs were SPF, certain species of

bacteria could be recovered from nasal and rectal swabs (Mr. D. Thompson personal communication). It is possible that the lambs in this experiment were colonised earlier or more heavily with bacteria than those in Experiment 1, a situation which might have stimulated increased immunoglobulin levels.

FCA stimulated high serum IgG levels which paralleled the steep rise in antibody titre and fractionation confirmed that the bulk of antibody was associated with this immunoglobulin. Fractionation of the nasal secretions from lambs which had been immunised IM with FCA resolved the anti-viral activity into 2 peaks. From other results (Sections III and IV) most of the first peak of activity was probably due to non-specific inhibitors, although IgM antibody may also have been involved. However the remaining peak of activity was associated with IgG_1 and even after concentration no activity was found in fractions containing IgA.

It is known that IgG_1 is selectively transferred from serum into the colostrum and milk of the ruminant (MacKenzie and Lascelles, 1968; Murphy et al, 1964) and recently Watson and Lascelles (1973 b) have demonstrated that IgG_1 is selectively transported into sheep saliva. Using radiolabelled immunoglobulins similar results have been obtained in the bovine with tears (Pedersen, 1973) and various other secretions, including respiratory fluids (Curtain et al, 1971). From this evidence it seems probable that the effect of FCA in this experiment was to stimulate very high circulating IgG antibody levels and that IgG_1 antibody was selectively transferred into the nasal secretions. However part of the IgG in the nasal secretions may have been locally produced, since pieces of bovine nasal mucosa cultured in vitro with radiolabelled amino acids have synthesised IgG (Butler et al, 1972).

EXPERIMENT 3

Introduction

The last experiment showed that high serum and nasal antibody titres could be stimulated and protection conferred in lambs either, 1) by two IM vaccinations with inactivated PI_3 in FCA, or 2) by one IM vaccination with inactivated PI_3 in FCA followed by IN instillation of inactivated virus without adjuvant. The object of this third experiment was to find out whether a single IM vaccination of inactivated PI_3 in FCA would be protective.

An anaphylatic like response occurred in the last experiment in those lambs which had been vaccinated with FCA. However it was not clear whether viral antigens were involved. In order to reduce the presence of non-viral antigens, the virus used for vaccination and challenge in this experiment was propagated in serum-free medium. The possibility of an anaphylactic reaction occurring after challenge as a result of hypersensitivity to non-viral antigens, was controlled by vaccinating a second group of lambs with virus-free medium emulsified in FCA.

Scheme of Experiment

Eight 10 day old, Blackface SPF lambs were randomly placed into 2 equal sized groups numbered 11 and 12. Lambs in Group 11 were vaccinated with control fluid and Group 12 was vaccinated with inactivated PI_3. Both vaccines were emulsified with an equal volume of FCA and were injected in 2 ml volumes IM into each lamb's left hind leg. Three weeks after vaccination both groups of lambs were challenged by exposure to aerosol.

A pool of infective material containing 10^7 TCID 50/ml of PI_3 was prepared as before except that serum was omitted from the culture medium. Control fluid was prepared from uninfected Roux cultures

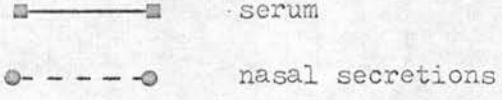
maintained in parallel with the same medium. Aliquots of the virus pool were inactivated as described previously. Aliquots of the virus pool which had not been inactivated were nebulised to produce the challenge aerosol. As in previous experiments the lambs were swabbed for virus isolation and clinically examined for 10 days after the challenge. Weekly blood and nasal secretion samples were collected from the lambs and assayed for virus neutralising antibody and immunoglobulins. A pool of nasal secretions was fractionated as described previously.

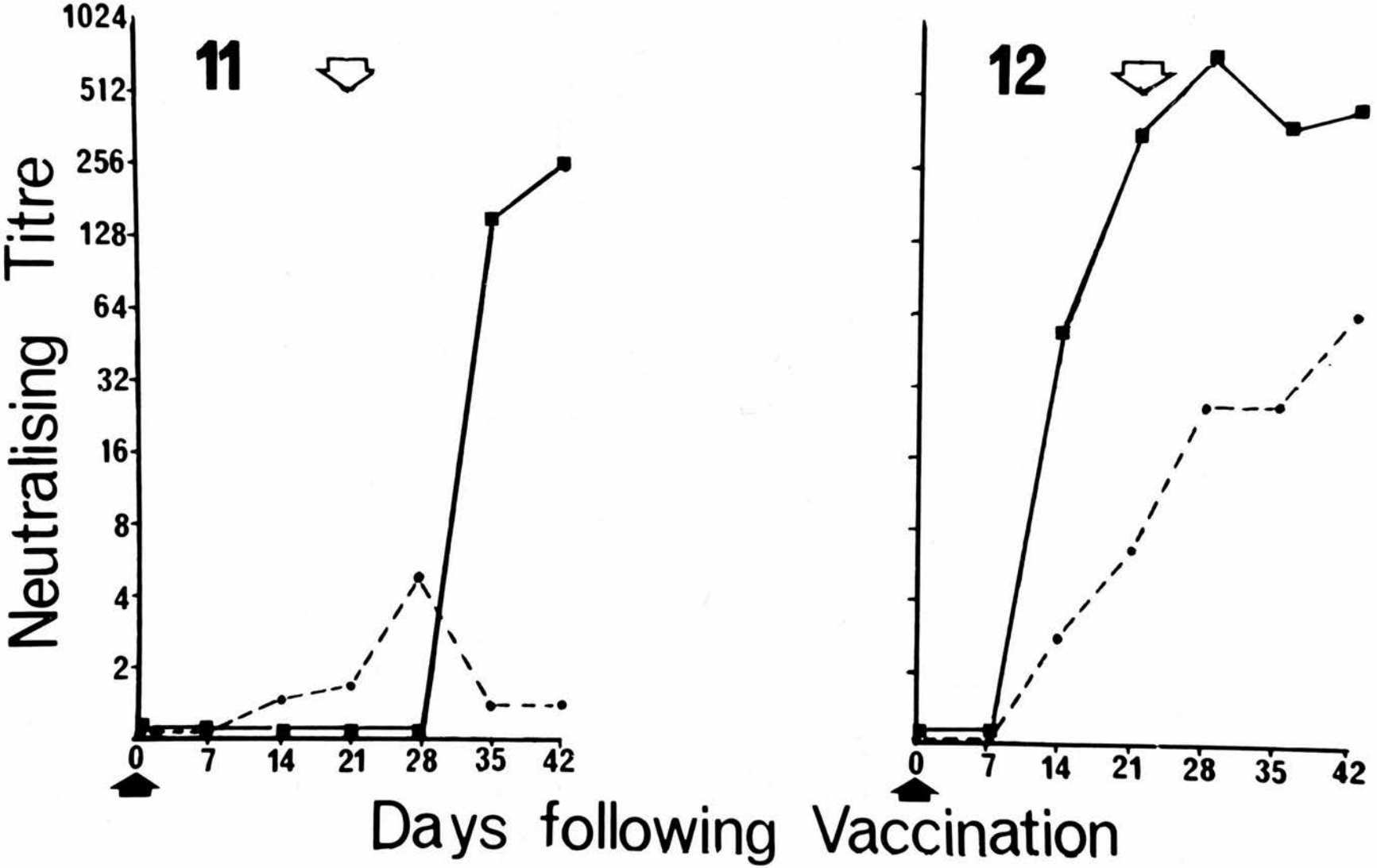
Results

Response to vaccination. In Group 12 serum antibody was first detected 14 days after vaccination at a mean titre of 60 increasing to almost 512 by the day of challenge (Fig. 35). Nasal antibody was also observed on these days at mean titres of approximately 3 and 6 respectively. No antibody was detected in the sera taken from Group 11 lambs before challenge although low levels of anti-viral activity were noted in the nasal secretions on days 14 and 21.

Response to challenge. Two point five ml of infective fluid containing $10^{7.5}$ TCID 50 virus/ml were nebulised during each exposure period. However no virus was recovered from a 10 minute impinger sample collected when Group 11 was being challenged. No clinical signs were noted after this challenge and no virus was recovered from the nasal swabs from any of the lambs. However a mean serum antibody titre of about 128 was detected 14 days after challenge in the control group (No. 11) and this increased almost 2 fold by day 42 of the experiment. In the nasal secretions of this group antibody increased temporarily to a mean titre of about 5 before falling to the low pre-challenge levels. In Group 12 meanwhile, mean serum titres increased 2-fold 7 days after the challenge but declined on days 35 and 42 to about the pre-challenge

Fig. 35 Group mean serum and nasal secretion antibody titres.




11
12
Neutralising Titre
1024
512
256
128
64
32
16
8
4
2
0
7
14
21
28
35
42
Days following Vaccination

level (400). A continuous rise in nasal secretion antibody was noted after challenge in this group and mean titres reached 64 by the end of the experiment (Fig. 36).

Immunoglobulins participating in the response.

a) Serum. As in the 2 previous experiments IgM concentrations were higher than IgG in the first serum samples examined although the absolute concentrations of both proteins were considerably greater than in previous groups at mean levels of about 175 and 75 mg/100 ml respectively (Fig. 36). After the administration of FCA IgG concentrations in all the lambs increased rapidly to group mean levels of some 400 mg/100 ml on the day of challenge. IgM levels however declined at similar rates in both groups as the experiment progressed. Following challenge an increase in IgG levels was observed in Group 12 but not in Group 11. IgA was not detected in any sera in this experiment.

b) Nasal Secretions. Except for a few samples in which very low levels were found, immunoglobulin was not detectable in the first nasal secretion samples collected from these lambs (Fig. 37). IgA concentrations soon increased and from day 21 onwards were at similar mean levels in both groups of lambs (5 to 10 mg/100 ml). The group mean concentrations of IgM were also similar, but at lower levels than IgA, fluctuating between 1 and 5 mg/100 ml. IgG was the major nasal immunoglobulin in both groups by 14 days after vaccination and concentrations were similar in both groups before challenge. Two weeks after the aerosol challenge however, a marked increase was observed in Group 12 IgG concentrations compared with Group 11, when the respective mean levels were estimated as 9 and 34 mg/100 ml.

Fig. 36

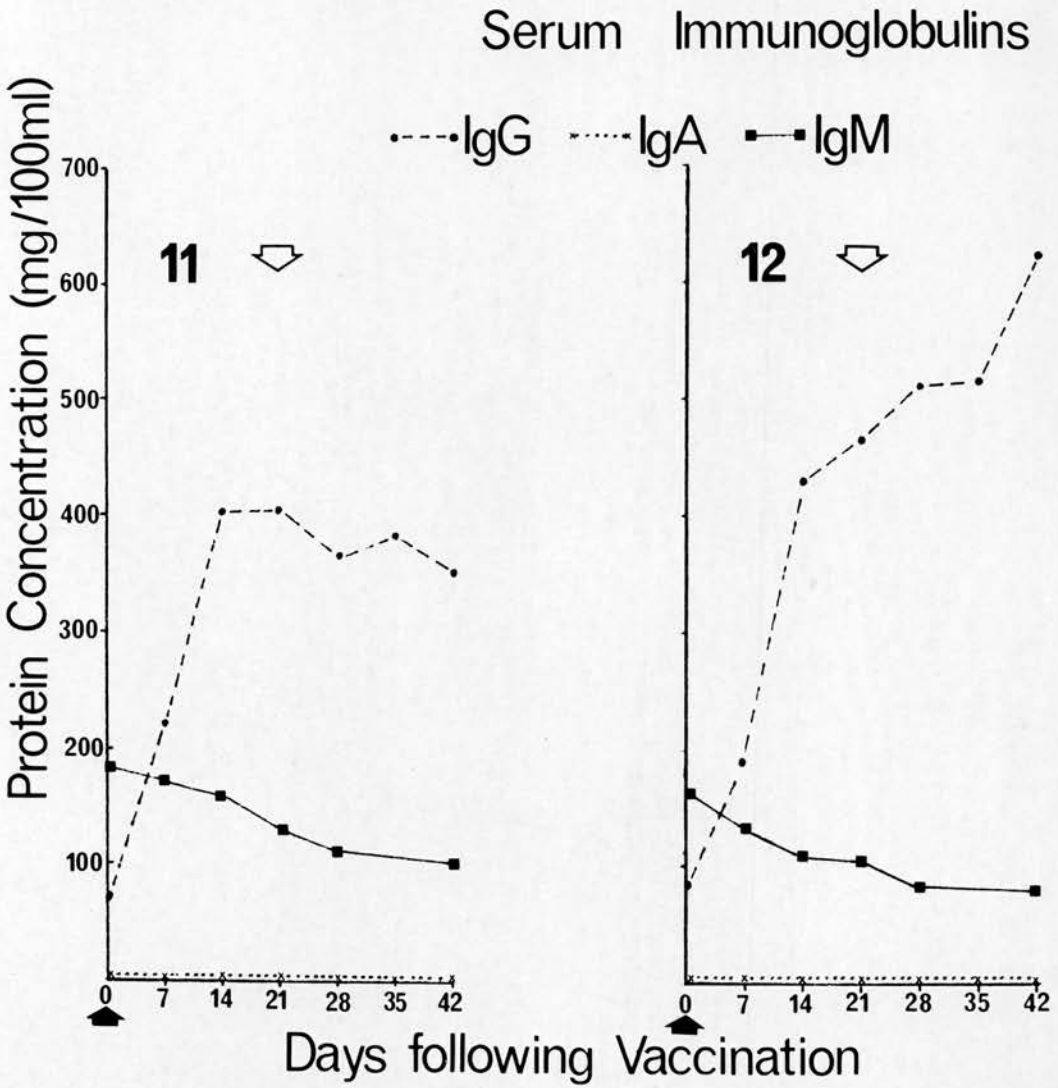
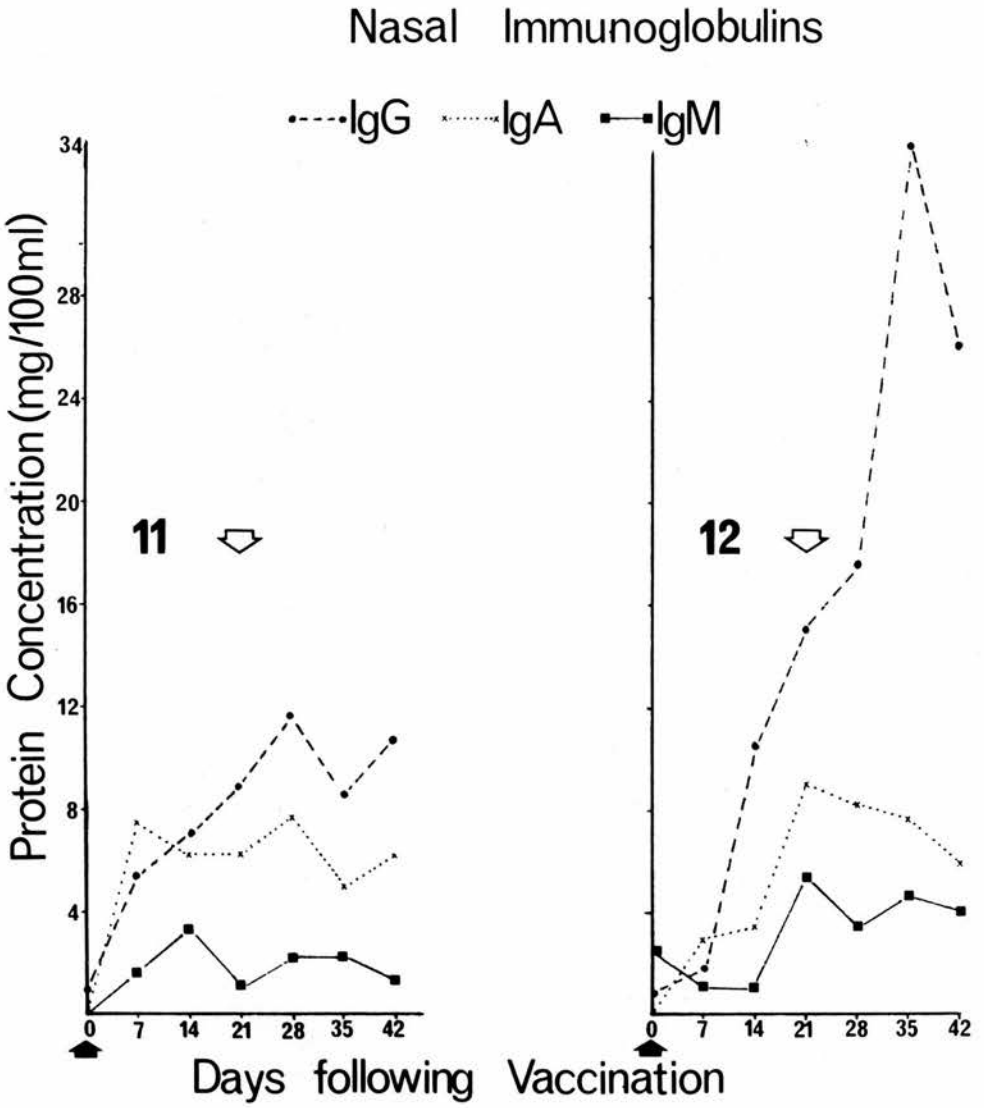

Serum Immunoglobulins
IgG
IgA
IgM
Protein Concentration (mg/100ml)
700
600
500
400
300
200
100
11
12
0
7
14
21
28
35
42
Days following Vaccination

Fig. 37


Nasal Immunoglobulins
IgG
IgA
IgM
Protein Concentration (mg/100ml)
34
28
24
20
16
12
8
4
11
12
0
7
14
21
28
35
42
Days following Vaccination

Pre-challenge nasal secretions from group 12 were pooled and filtered through the Biogel column. HI activity was separated into 2 peaks (Fig. 38). The first peak was eluted at the exclusion volume, in association with IgM. The second peak coincided with the IgG fractions. Little activity was associated with the IgA containing fractions.

Discussion

The nasal and serum antibody response following vaccination of Group 12 lambs was similar to that following the first vaccination of Groups 7 and 9. Some neutralising activity was observed in the nasal secretions from Group 11 lambs, but this activity was so low that it was of doubtful significance.

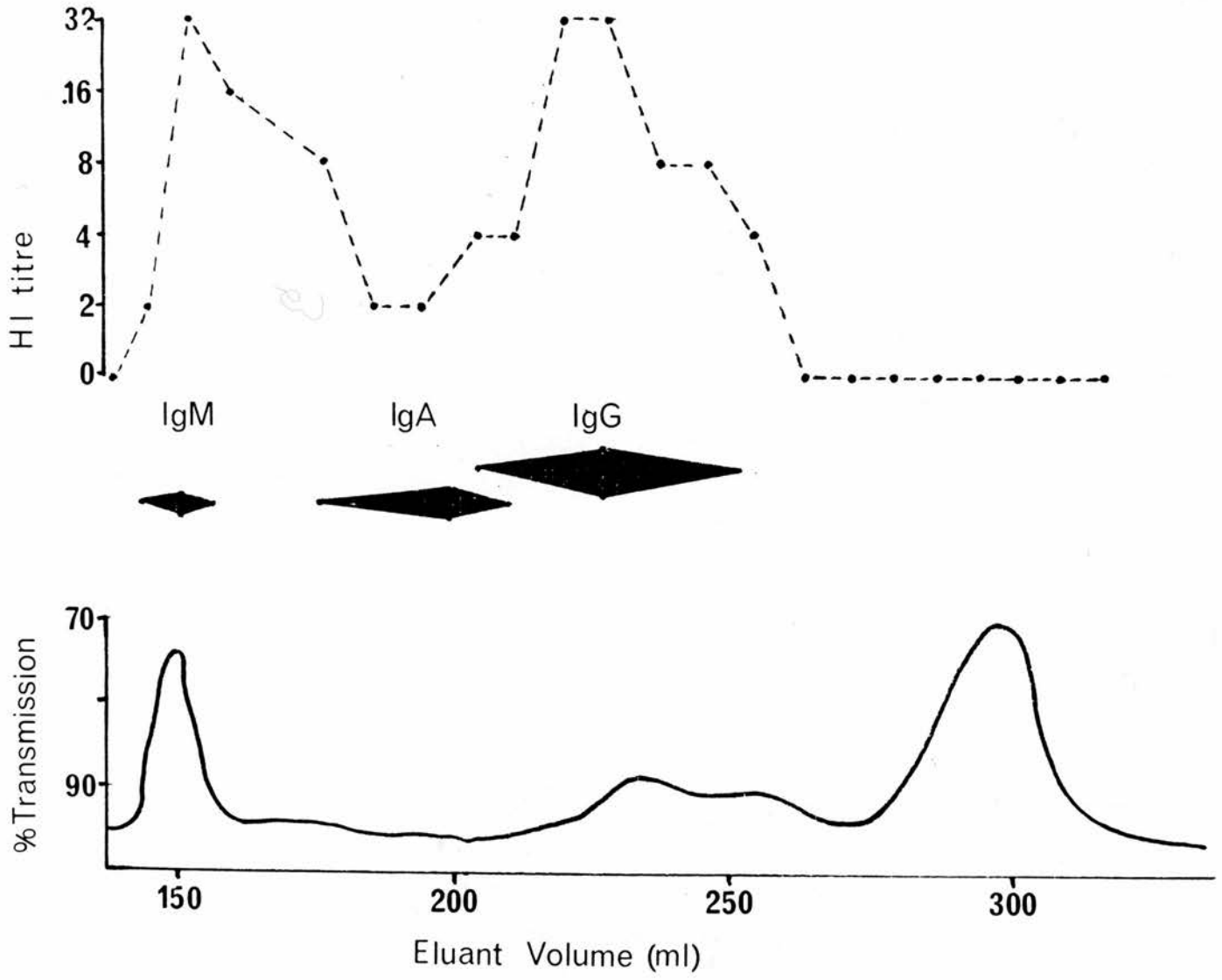
Since no virus was recovered in the impinger sample or from the nasal swabs of the control lambs , it appeared that the challenge aerosol had failed. However the sharp increase of serum antibody in Group 11 after challenge clearly indicated that the control lambs had inhaled virus antigen, which presumably was not infective. It seems likely that the virus was inactivated during or after nebulisation. The reason for this is not known; possibly PI_3 virus in serum-free medium is unable to withstand nebulisation. Since virus was not recovered from the control group, it was obviously not known whether the vaccination would have protected Group 12. Although virus antigen was inhaled after challenge, no anaphylactic type reaction was observed in any lamb. This suggests that the reactions seen in Experiment 2 may not have been caused by hypersensitivity to viral proteins.

Following IM administration of FCA, IgG became the major nasal secretion immunoglobulin as found in Experiment 2. As previously discussed, the high nasal secretion IgG levels may have been due to selective transport of IgG_1 from the serum. Fractionation of the nasal secretions also produced identical results to those in Experiment 2,

Fig 38 Fractionation of nasal secretions by filtration through Biogel A 1.5 M.

Sample: 4 ml of nasal secretions collected from Group 12 before challenge.

Column: 70 x 2.5 cm. Fraction size: 11 ml.


32
16
8
4
2
0
HI titre
IgM
IgA
IgG
70
90
% Transmission
150
200
250
300
Eluant Volume (ml)

with all the antibody activity confined to the IgG containing fractions. The first peak of HI activity was probably due to non-specific inhibitors (see part 1 of this Section and Section IV, part 2).

Of interest was the finding that the post challenge serum titres in the control group were very high, similar to those following vaccination of Group 12. Thus, when FCA was inoculated 3 weeks before the antigen and by a different route, the serum antibody response was comparable to that following simultaneous administration of antigen and adjuvant at the same site. However no such similarity was observed in the nasal secretion antibody response. If the antibody in the nasal secretions was largely derived from serum, why, in the presence of similar serum antibody titres, were the post challenge nasal secretion titres in Group 11 lower than the post vaccination nasal titres in Group 12? One possible explanation is that following vaccination of the immunologically virgin Group 12 lambs, a relatively high proportion of serum IgG_1 would have anti-PI_3 specificity, whereas following challenge of Group 11 this proportion would be lower. Therefore in Group 11 more IgG_1 molecules of other specificity would be available in the serum to compete for selective transport into the nasal secretions, causing a reduced nasal secretion titre.

SECTION IV

PART 1

The Immune Response of Newborn Suckled Lambs following Intranasal Challenge with Parainfluenza 3 Virus.

Introduction

The results of the vaccination/challenge experiments with SPF lambs in Section III showed that the presence of antibody either in the serum or nasal secretions correlated closely with protection against infection with PI_3. However the role if any of cell mediated immunity was not known. The object of the experiment described in Part I of this section was to find out whether lambs passively immunised with antibody would be protected. The most convenient and most natural situation to examine was to challenge newborn lambs which had colostral antibody. Unless infected *in utero* or very early in life, these animals could not have any active immunity to the virus and therefore any protection conferred could be attributed to the action of antibody alone. In order to reduce the chance of neonatal infection the lambs were removed from their dams before they were 1 week old and housed in isolation. Nasal secretions were also collected from these animals to investigate whether colostral antibody could be detected in the nasal secretions.

Materials and Methods.

Lambs. Eight conventionally reared Suffolk lambs, aged between 3 and 7 days, were placed in 2 groups according to their dams' serum HI titres, assayed approximately 2 weeks before parturition. Four lambs from 3 ewes with serum HI titres less than 10 and 4 lambs from 3 ewes with titres of 20 or greater were placed into Groups A and B respectively. Each group was housed in a separate loose box and reared on milk substitute*,

* Nutrilamb, Scottish Agricultural Industries, Ltd.

which contained no detectable immunoglobulin. Protein concentrates and hay were also available ad lib.

Experimental Scheme. One ml of fluid containing $10^{6.3}$ TCID 50 of PI_3 was instilled into each nostril, when the lambs were between 5 and 9 days old. Virus isolation was attempted from nasal swabs, collected from the right nostril just before and for ten days after inoculation with virus. Blood and nasal secretion samples were collected on 2 occasions before challenge, and on days 2, 4, 7, 10, 14 and 21 after challenge. Nasal secretions were collected from the left nostril. All samples were stored at -20°C before virus neutralising and immunoglobulin assays were performed.

Results

The results of virus recovery and the serum and nasal antibody titres are shown in Table 12.

Pre-challenge antibody. Although their dams had serum HI titres less than 10, two of the lambs in Group A (Nos. 403 and 631) had low serum neutralising titres prior to challenge. However antibody was not detected in the nasal secretions of these lambs. Group B lambs all had higher serum titres than Group A before challenge, especially lamb 402 which had a titre of 2048. Although Nos. 407 and 408 were twins, a 6 fold difference in the serum antibody titres was found. Pre-challenge nasal antibody was also detected in the 3 Group B lambs which had the highest serum titres.

Virus Recovery. No virus was recovered from the nasal swabs taken a few minutes before inoculation. After the intranasal challenge no clinical signs were observed apart from the occasional mild nasal discharge. Virus was recovered on at least one occasion from all the lambs in Group A but only from two of the lambs in Group B. In Group B PI_3 was isolated twice from lamb 405 and on 7 days from lamb

Table 12.

Group	Lamb No.	Age at D-2 (days)	V[1]		Virus Neutralising Titre Days after Challenge -2	0	2	4	7	10	14	21
A	406	4	4	S[2]	0[4]	0	0	0	0	8	23	32
				N[3]	0	0	0	0	0	0	2	0
	631	6	7	S	6	6	NT	3	2	4	8	8
				N	0	0	0	0	0	0	0	0
T	403	5	8	S	6	3	11	8	11	11	23	32
				N	0	NT	0	0	0	0	0	0
T	404	5	1	S	0	0	0	0	2	8	45	32
				N	0	0	0	0	0	0	0	0
B	405	3	2	S	45	91	45	32	45	45	32	32
				N	16	8	4	4	3	8	6	4
T	407	4	0	S	91	64	23	32	32	32	45	16
				N	4	3	0	0	0	0	0	0
T	408	4	7	S	NT	11	11	11	32	16	64	45
				N	0	0	0	0	0	0	0	0
	402	7	0	S	2048	2048	2048	2048	2048	2048	2048	2048
				N	64	64	NT	8	2	16	6	8

V[1] = No. of times virus recovered; S[2] = Serum; N[3] = Nasal Secretions;

O[4] = 2; NT = Not Tested; T = Twins.

408, which had the lowest serum and nasal antibody titres of the group. However virus was only re-isolated once from lamb 404, although no pre-inoculation antibody activity was detected in the serum or nasal secretions of this lamb.

Post-challenge antibody. After challenge a 4 fold or greater rise in serum antibody was observed in 3 lambs in Group A and one lamb in Group B Except for lamb No. 404, virus had been recovered on at least 4 occasions from these lambs. No. 631 was also surprising since no significant rise in serum antibody was detected, despite the fact that virus was isolated 7 times from this lamb. Nasal antibody was only detected in one post-challenge sample from the lambs which did not have pre-challenge nasal antibody.

Immunoglobulins in Serum and Nasal Secretions.

Serum. There was considerable variation in the concentration of serum immunoglobulins in different lambs. This presumably was due to variation in the amount of colostrum ingested. Thus lamb 407 had about 4 fold higher levels of all 3 immunoglobulins than its twin. Lamb 631, which was one of quadruplets, had the lowest IgG and IgM levels at the start of the experiment (Tables 13, 14 and 15).

IgG constituted about 90% of the serum immunoglobulins and IgG levels declined during the 3 week period of the experiment. The decline in serum IgG was less marked in the 2 lambs with the lowest initial IgG concentrations (Nos. 631 and 408). Serum IgM levels also showed a downward trend in most lambs as the experiment progressed. However serum IgM levels in lamb 631 increased by the end of the experiment. IgA was detected in 6 lambs' sera collected 2 days before challenge. However serum IgA rapidly disappeared and was not detected later than 2 days after challenge.

Nasal Secretions. High levels of IgG were found in the lambs' nasal

Table 13.

IMMUNOGLOBULINS IN SERUM MG/100 ML

Group	Lamb Number	Age at D-2 (days)	IgG							
			Days After Challenge							
			-2	0	2	4	7	10	14	21
A	406	4	6500	3900	2500	2750	4000	4200	1900	1500
	631	6	500	320	ND	290	330	ND	320	320
Twins	(403	5	2400	2000	2100	1600	1450	1450	1400	1100
	(404	5	1800	1700	1750	1600	1700	1700	1200	1000
B	405	3	1700	1300	1150	1300	1250	1250	680	390
Twins	(407	4	3000	4500	4200	4500	2350	1700	2150	1700
	(408	4	810	780	780	690	ND	430	810	600
	402	7	2000	1700	1900	2000	2100	1900	1700	1650

IMMUNOGLOBULINS IN NASAL SECRETIONS MG/100 ML

A	406	4	170	150	54	80	100	66	50	24
	631	6	10	19	6.6	11	13	19	ND	6
Twins	(403	5	390	130	38	ND	54	52	30	22
	(404	5	250	80	45	23	14	17	17	4.7
B	405	3	ND	80	84	50	21	13	23	17
Twins	(407	4	230	110	130	95	64	66	39	43
	(408	4	54	54	ND	21	20	13	12	10.5
	402	7	230	95	150	90	26	66	27	47

ND = not done

Table 14.

IMMUNOGLOBULINS IN SERUM MG/100 ML

Group	Lamb Number	Age at D-2 (Days)	IgM							
			Days after Challenge							
			-2	0	2	4	7	10	14	21
A	406	4	350	320	200	180	170	200	78	52
	631	6	23	27	ND	98	45	119	45	60
Twins	403	5	230	180	190	126	144	105	112	60
	404	5	190	119	105	98	126	83	56	25
B	405	3	116	51	49	35	32	22	15	10
Twins	407	4	420	420	170	270	170	105	98	85
	408	4	128	102	78	56	43	37	30	34
	402	7	170	210	119	86	76	71	102	42

IMMUNOGLOBULINS IN NASAL SECRETIONS MG/100 ML

A	406	4	6.1	4.5	4.7	0	ND	0	3.9	0
	631	6	ND	0	0	0	0	8.1	ND	0
Twins	403	5	5.1	3.7	0	ND	0	3.7	0	5.7
	404	5	7.1	0	0	0	0	0	0	0
B	405	3	8.8	0	0	6.3	0	0	0	0
Twins	407	4	17	6.8	9.5	6.1	9.5	5.9	5.2	ND
	408	4	0	0	0	0	3.7	3.9	4.2	0
	402	7	9.5	6.8	9.5	5.1	8.4	4.2	0	3.7

ND = not done

Table 15.

IMMUNOGLOBULINS IN SERUM MG/100 ML

Group	Lamb Number	Age at (D-2 (Days)	IgA							
			Days after Challenge							
			-2	0	2	4	7	10	14	21
A	406	4	22	8	4.8	0	0	0	0	0
	631	6	9.6	4.6	0	0	0	0	0	0
Twins	403	5	0	0	0	0	0	0	0	0
	404	5	7.2	0	0	0	0	0	0	0
B	405	3	9.6	0	0	0	0	0	0	0
Twins	407	4	46	13	5.8	0	0	0	0	0
	408	4	12	0	0	0	0	0	0	0
	402	7	0	0	0	0	0	0	0	0

IMMUNOGLOBULINS IN NASAL SECRETIONS MG/100 ML

A	406	4	0	0	0	0	0	6.0	4.8	4.1
	631	6	ND	0	0	0	6.0	6.0	ND	4.7
Twins	403	5	0	0	0	ND	0	0	0	0
	404	5	0	0	0	0	0	0	0	0
B	405	3	0	0	0	0	0	0	6.0	6.0
Twins	407	4	4.2	0	0	0	3.8	4.2	6.0	ND
	408	4	0	0	0	0	0	6.7	8.7	6.7
	402	7	0	0	5.5	5.1	7.9	10.4	7.2	8.7

ND = not done

secretions. Nasal secretion IgG levels declined as the lambs grew older. IgM was detected at low concentrations in pre-challenge nasal secretions from 6 lambs. In 2 lambs (402 and 407) IgM was detected regularily, but in the remaining animals the levels were usually below the limits of detection. IgA was found in one nasal secretion sample prior to challenge. However IgA was detected regularily in the nasal secretions of 6 lambs aged 2 weeks or more. On the other hand, no IgA was found in the secretions of one pair of twin lambs (Nos. 403 and 404) throughout the experiment.

Discussion.

The number of lambs involved in this experiment was too small to draw any firm conclusions. However it seemed that high titres of passively acquired nasal secretion and serum antibody could protect against PI_3 infection. Thus virus was recovered on only 2 occasions from the 3 lambs which had minimum pre-challenge serum and nasal titres of 64 and 3, whereas 27 isolations were made from 5 lambs which had maximum serum titres of 11 and no detectable nasal antibody. In addition 4 of these 5 lambs showed a 4 fold rise in serum antibody after challenge, while no increase was detected in the 3 animals with the high pre-challenge titres. Thus all four of the lambs taken from ewes with undetectable serum titres were susceptible to the virus challenge, while only one of the 4 lambs from dams with serum antibody was susceptible. The fact that this susceptible lamb (No. 408) had an immune sibling shows that the quantity as well as the quality of colostrum absorbed is important. Thus, judging by antibody titres and immunoglobulin levels, lamb 408 must have ingested about 1/5 the amount of colostrum received by its twin (407), rendering it susceptible to the challenge infection while its sibling was immune.

The reason for the lack of detectable nasal antibody in these

lambs after infection with PI_3 is not known. Nasal antibody was previously detected in colostrum deprived lambs of this age after infection with virus (Section III, part 2). Possibly the presence of maternal antibody inhibited active production of nasal antibody. However the result needs to be confirmed before such speculation can be substantiated.

Of considerable interest was the finding that high levels of IgG, greater than adult concentrations in some cases (Section II), were present in the nasal secretions, with nasal antibody also present in those lambs which had the highest serum titres. Although no pre-suckling samples were taken, it is unlikely that the very high IgG levels in the nasal secretions were due to active production by the lambs either in utero or early in life. The lambs in this experiment had been weaned from the ewes and reared on substitute milk containing no detectable immunoglobulin. Thus the source of nasal secretion IgG was not from milk inhaled into the nasal secretions. It seemed much more likely that the IgG in the nasal secretions was passively acquired after transudation from the serum through the newborn lamb's nasal mucosa. This proposal was substantiated by the finding that nasal secretion IgG levels fell as the lambs grew older, paralleling the situation in the serum. In contrast, the nasal secretions collected from more than 50 colostrum deprived lambs of similar age (Section III, part 2) contained very low levels of IgG which gradually increased as the lambs grew older.

No occult blood was detected in the nasal secretion samples tested. Nevertheless the possibility existed that the apparent transudation of IgG from the serum into the secretions was caused by trauma to the delicate nasal mucosa of the newborn lamb. This possibility was tested in the next part of this thesis.

PART 2

Maternal Immunoglobulins and Parainfluenza 3 Virus Inhibitors in the Serum and Nasal Secretions of Newborn Lambs.

Introduction

In the first part of this section immunoglobulins and PI_3 antibodies were found in the nasal secretions of newborn suckled lambs. It seemed likely that this immunoglobulin was of colostral origin. However nasal secretions had not been collected before the lambs sucked. In addition, the possibility had not been ruled out that the nasal secretion IgG had transuded from serum following trauma to the delicate nasal mucosa by the tampons.

The experiment to be described was designed to examine in more detail the possibility of colostral derived immunoglobulin reaching the nasal secretions. Samples were collected from a total of 34 lambs, which were not inoculated with PI_3. Attempts were made to obtain pre-suckling samples of all the fluids collected. Nasal washings, as well as nasal secretions obtained by tampons were examined for immunoglobulins. In addition naturally reared lambs were compared with lambs which had ingested colostrum and which were subsequently reared on immunoglobulin-free milk substitute. This was to ascertain whether sucking natural milk altered the immunoglobulin content in the lambs' nasal washings. Samples of lachrymal fluid were obtained from some lambs to discover whether passively acquired immunoglobulin was present in this fluid. Changes in the immunoglobulin concentrations were measured in serial serum samples from the lambs up to 3 weeks of age and thus the half lives of colostral IgG, IgM and IgA were estimated.

Materials and Methods

Ewes and Lambs. Thirty four lambs which were the offspring of 19 ewes were examined in this experiment. Ten pairs of twins and 4 single lambs from 14 Suffolk ewes were sampled while sucking their dams. Five pairs of twin lambs from 5 Scottish Blackface ewes were allowed to suck for one day and then were removed from the ewes and housed indoors. These lambs were fed milk substitute which contained no detectable immunoglobulin.

Sampling. Colostrum was obtained before suckling. Serum was harvested from up to 5 ml of blood which was withdrawn from the jugular vein. Nasal secretions were collected with small pieces of tampon as before (Section II, part 2). Care was taken to avoid trauma to the nasal mucosa. Usually 0.1 ml to 0.5 ml of fluid was obtained after the tampon had been squeezed out in a 1 ml syringe. Nasal washings were collected by instilling 5 ml of PBS into each nostril and collecting the expelled fluid in a universal bottle. Between 3 and 8 ml of fluid was recovered from each lamb. Each nasal washing sample was concentrated 10 fold by dialysis against polyethylene glycol (20,000 MW). Tears were collected as previously described (Section II, part 2).

Samples of nasal secretions were tested for occult blood and all samples of each fluid were stored at -20°C until assayed for immunoglobulin content.

Scheme of Experiment (Table 16). Pre-suckling samples of each body fluid were either obtained within half an hour of birth or else the lambs were removed from their dams at this time and sampled within three hours. Blood and colostrum samples were obtained from most of the ewes at this time. Thereafter samples of each fluid were collected on days 1, 3, 5, 7, 10, 14 and 21 after birth, except that day 10

Table 16.

Scheme of Experiment

Group	Breed	Number of Ewes	Number of Lambs	Samples Collected	Method of Rearing
A	Suffolk	8	14	Blood & nasal secretions	Natural
B	Suffolk	6	10	Blood & nasal washings	Natural
C	Scottish Blackface	5	10	Blood & nasal washings and lachrymal fluid	Artificial

samples were not obtained from the Scottish Blackface lambs (Group C). Nasal secretions were obtained from 14 of the Suffolk lambs (Group A), whereas nasal washings were collected from the remaining 10 Suffolk lambs (Group B) and from the Blackface lambs (Group C). Lachrymal fluid samples were obtained from Group C only.

Results

Mean immunoglobulin concentrations are presented in the text with their Standard Error.

Ewe sera and colostra. The mean immunoglobulin concentrations in the ewe sera were: IgG 32.7 ± 4.5 mg/ml; IgM 1.72 ± 0.14 mg/ml and IgA 0.17 ± 0.02 mg/ml. In the colostrum the mean concentrations were: IgG 101.2 ± 7.2 mg/ml; IgM 2.9 ± 0.2 mg/ml and IgA 6.2 ± 0.9 mg/ml (Table 17). No correlation was observed in serum and colostral immunoglobulin concentrations of the 12 ewes from which both samples were collected (Table 18).

Lamb sera. Low levels of IgM were detected in the pre-suckling serum samples from all the lambs (Table 19). IgA was present in 4 of these sera, whereas IgG was found in 15 samples. IgA and IgG levels were low, except for lamb 320, which had a serum IgG concentration of 70 mg/ml.

Maternal immunoglobulins reached peak levels in the lamb sera on day 1, thereafter declining over the 3 week period of monitoring. There was considerable variation in the peak levels in individual lambs (Table 17). Six of the lambs (Nos. 306, 307, 316, 319, 1680 and 1681) had serum IgG levels less than 20 mg/ml and these animals were considered hypogammaglobulinaemic. Excluding these 6 lambs, the mean serum immunoglobulin concentrations on day 1 were: IgG 47.9 ± 3.9 mg/ml, IgM 3.03 ± 0.41 mg/ml and IgA 1.62 ± 0.25 mg/ml

Table 17.

Immunoglobulin Concentrations (mg/ml).

Ewe No.	Colostrum			Lamb No.	Serum (day 1)		
	IgG	IgM	IgA		IgG	IgM	IgA
10399	108	2.4	6.2	301	64.0	3.6	2.0
				302	36.5	2.3	0.7
10559	164	4.1	7.9	303	48.0	3.4	1.3
				304	94.0	9.6	2.6
10679	96	1.5	5.8	305	45.0	1.6	2.3
				306	8.8	0.3	0.1
10500	96	1.8	4.0	307	0.9	0.2	0
10328	78	0.8	6.2	308	75.0	0.8	2.6
				309	50.0	0.7	2.6
A789	104	2.2	2.4	311	75.0	3.3	1.3
10675	66	2.2	3.3	3.2	33.0	0.7	1.1
				313	47.0	1.4	0.7
10334	129	3.2	5.8	316	8.2	0.5	0
				317	21.5	1.7	0.7
10677	93	4.1	1.5	318	29.5	2.0	0.3
				319	11.0	1.1	0
10501	66	3.0	3.3	320	44.15	6.8	0.6
				321	64.0	3.8	0
10555	84	2.1	6.7	324	49.0	2.2	3.2
				325	71.0	5.8	1.3
10355	50	3.8	3.3	328	39.15	5.5	1.3
				329	62.0	3.0	0.5
A775	96	3.2	3.9	330	92.0	5.8	0.9
10409	93	3.4	4.6	331	47.0	4.8	1.0
				1670	38.0	1.5	5.8
9781	168	4.5	17.3	1671	26.0	1.1	3.0
				1672	21.0	0.9	1.1
5V26	102	3.6	16.1	1673	34.0	1.5	3.6
				1674	67.0	4.5	3.0
B541	72	2.6	4.3	1675	52.0	5.1	1.3
				1680	2.5	0.1	0
B543	120	3.2	10.5	1681	10.0	0.5	0
				1682	27.0	2.1	0.5
B563	138	3.8	5.6	1683	25.0	1.4	NT

NT - not tested

Table 18.

Immunoglobulin Concentrations mg/ml.

Group	Ewe No.	Colostrum IgG	Colostrum IgM	Colostrum IgA	Serum IgG	Serum IgM	Serum IgA
A	10399	108	2.4	6.2	31	2.3	0.2
	10599	164	4.1	7.9	33	1.4	0.3
	10679	96	1.5	5.8	34	0.9	0.1
	10500	96	1.8	4.0	45	1.2	0.1
	10328	78	0.8	6.2	68	2.1	0.3
	A789	104	2.2	2.4	39	2.1	0.1
	10675	66	2.2	3.3	29.5	2.1	0.2
	10334	129	3.2	5.8	38	1.6	0.2
B	10677	93	4.1	1.5	15	2.1	0.1
	10501	66	3.0	3.3	13.5	1.9	0.1
	10555	84	2.1	6.7	11	0.9	0.3
	10355	50	3.8	3.3	NT	NT	NT
	A775	96	3.2	3.9	NT	NT	NT
	10409	93	3.4	4.6	35	1.9	0

NT = Not Tested.

Table 19

Immunoglobulin Concentrations (mg/ml) in the Precolostral Lamb Sera

Group	Ewe No.	Lamb No.	IgG.	IgA	IgM
A.	10399	301	0;11	0	0.21
		302	0.14	0	0.11
	10559	303	0.05	0	0.03
		304	0	0	0.08
	10679	305	0.21	0	0.08
		306	0.05	0	0.06
	10500	307	0.88	0	0.25
	10328	308	0	0	0.11
		309	0	0	0.07
	A789	311	0.44	0	0.13
	10675	312	0	0	0.08
		313	0	0	0.07
	10334	316	0.05	0	0.10
		317	1.85	0.12	0.46
B.	10677	318	0	0	0.07
		319	0	0	0.07
	10501	320	70.00	0.24	0.34
		321	9	0	0.07
	10555	324	0	0	0.09
		325	0	0	0.07
	10355	328	1.50	0.05	0.72
	A775	329	0	0	0.13
		330	1.50	0	0.07
	10409	331	0	0	0.04
C.	9781	1670	0	0	0.27
		1671	0.05	0	0.34
	5V26	1672	0	0	0.04
		1673	0	0	0.04
	B541	1674	0.08	0.07	0.09
		1675	6.40	0	0.10
	B543	1680	0	0	0.04
		1681	0	0	0.13
	B553	1682	0	0	0.27
		1683	0	0	NT

NT - not tested.

Three of the 6 hypogammaglobulinaemic lambs had siblings with normal serum immunoglobulin levels; thus considerable variation could be found in the serum immunoglobulin concentrations of sibling lambs. Moreover in certain pairs of lambs (Nos. 312 and 313, 320 and 321, 324 and 325, 1674 and 1675), the variation in the levels of one immunoglobulin class did not parallel the variation in the other 2 classes (Table 17). The 4 single lambs sampled did not have noticeably higher serum immunoglobulin levels than individual twin lambs.

From maximum concentrations on day 1, the levels of maternal IgG, IgM and IgA declined at different rates in the lamb sera. Excluding the 6 lambs with the low initial levels, the changes in mean serum immunoglobulin levels over the 3 week period were plotted and calculation of the regression coefficients revealed the following half lives: IgG 13.7 days; IgM 4.1 days and IgA 1.8 days (Fig. 39).

Although one ewe (No. 10334) had an indurated mammary gland, no obvious reason was found to account for the remaining 5 hypogammaglobulinaemic lambs. One of these lambs died of an undiagnosed cause. Some of these lambs showed increases in serum IgM and IgG levels, indicating active synthesis of these immunoglobulins before three weeks of age.

Nasal secretions. No IgG was detected in the nasal secretions collected from newborn lambs prior to sucking but it was present in all samples obtained on day 1, approximately 24 hours after first sucking. Great individual variation in the concentrations was noted and there was considerable variation in samples taken from sibling lambs. Those lambs with lower serum IgG levels did not have appreciably lower levels of IgG in their nasal secretions. IgG concentrations reached a peak on day 1 and thereafter declined (Fig. 40).

Fig. 39 Serial changes in the serum concentrations of maternal immunoglobulins.

- - - - - - - regression lines.

I - standard error of the mean.

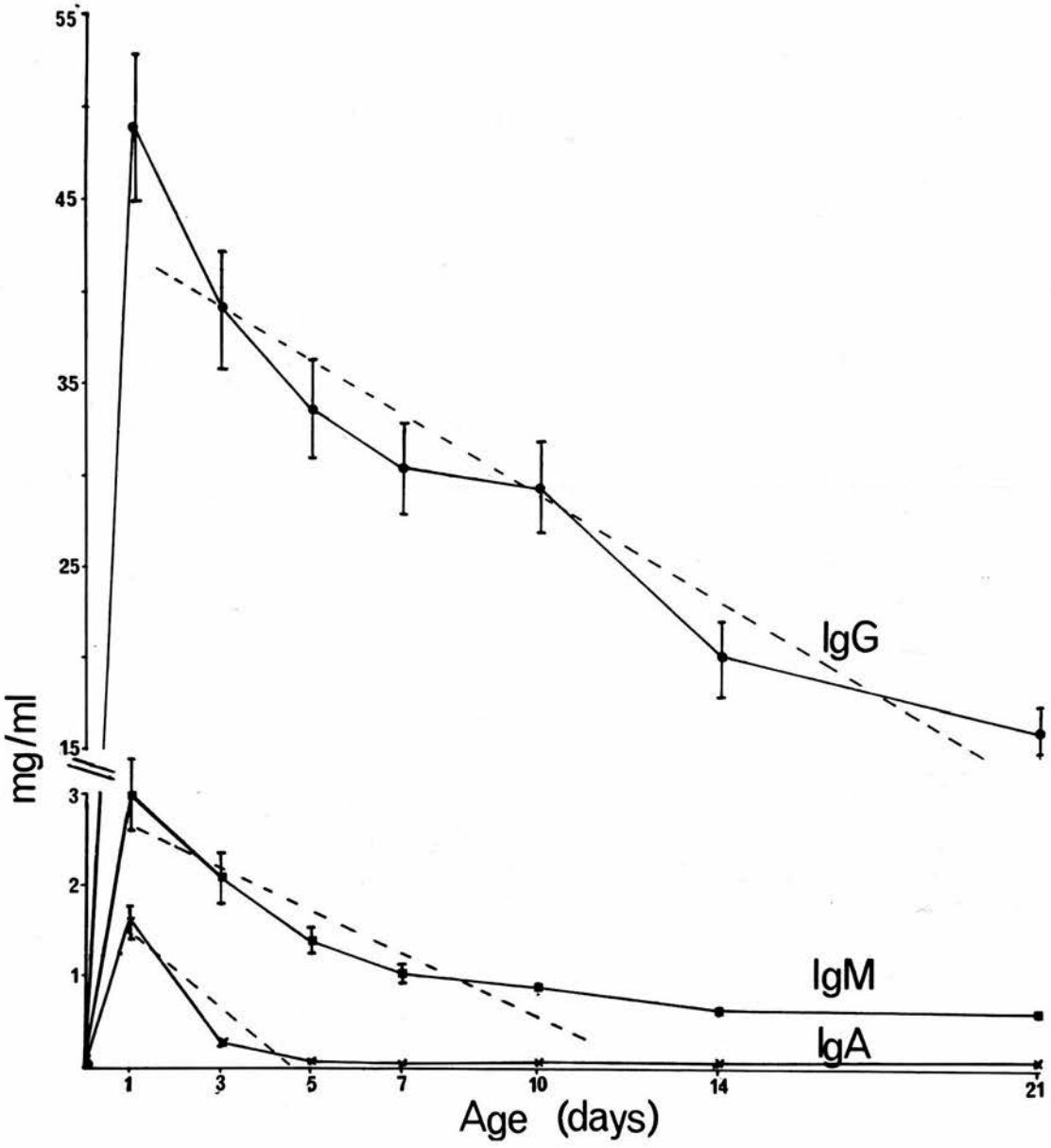
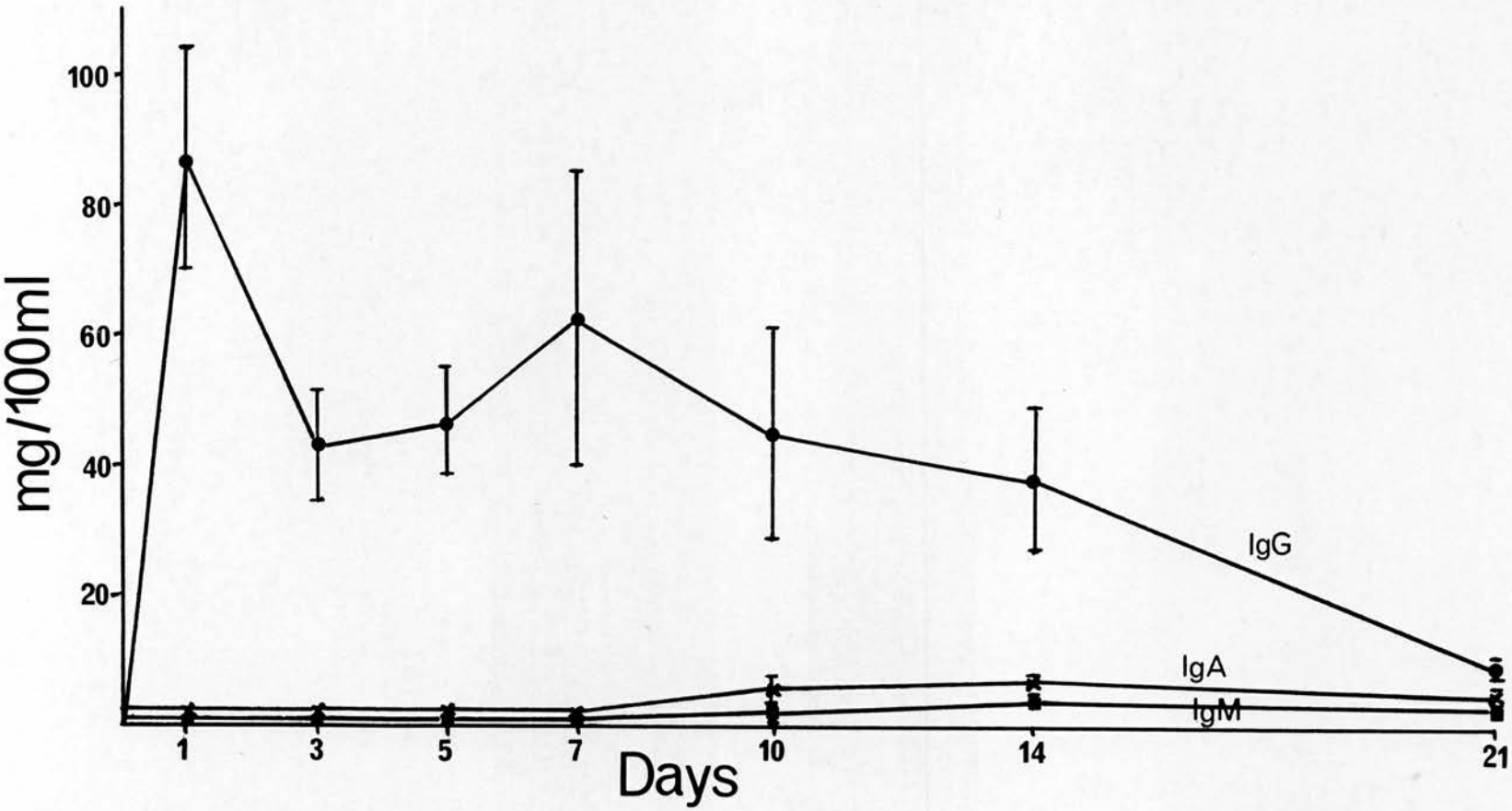

55
45
35
25
15
3
2
1
mg/ml
IgG
IgM
IgA
1
3
5
7
10
14
21
Age (days)

Fig. 40 Serial changes in the nasal secretion immunoglobulin concentrations of Group A lambs.

I - standard error of the mean.


mg/100ml
100
80
60
40
20
IgG
IgA
IgM
1
3
5
7
10
14
21
Days

IgA was not detected in any nasal secretion samples before day 5, when it was found in one lamb, but IgA was found in the nasal secretions of most lambs by day 14 (Fig. 40). IgM appeared in the nasal secretions about the same time as IgA.

Nasal Washings. Concentrations of immunoglobulins in nasal washings collected from Groups B and C were approximately twenty-fold less than those in nasal secretion samples. However, the qualitative changes observed over the period of monitoring were similar (Fig. 41). IgG was not detected in any precolostral samples but was consistently present (in both Groups B and C) after sucking. IgA and IgM were detected in nasal washings from most lambs by day 14, and in several lambs IgA was the predominant immunoglobulin in the nasal washings by 3 weeks of age.

Lachrymal Secretions. The changes in the mean concentrations of immunoglobulins in the lachrymal fluid of lambs in Group C are shown in Fig. 42. As in nasal secretions or nasal washings, immunoglobulins were not detected in precolostral samples, but after sucking IgG was present in all samples. IgA and IgM were not detected until lambs were 2 to 3 weeks old, but mean IgA levels exceeded mean IgG levels by 21 days after birth.

PI_3 Inhibitors in Serum and Nasal Secretions. Sera collected from 10 one day old lambs and their dams were titrated for HI antibody to PI_3. Within pairs of twin lambs individual serum HI titres and IgG concentrations correlated closely (Table 20) and relative to serum IgG levels, the antibody titre of each lamb tended to reflect that of its mother. However 3 lambs had titres of 10 or less. In 2 cases (Nos. 306 and 307) this was due to poor transfer of IgG from dams which had serum antibody. The third lamb (No. 311) had received a

Fig. 41 Serial changes in the immunoglobulin concentrations of the nasal washings collected from Groups B and C.

I - standard error of the mean.

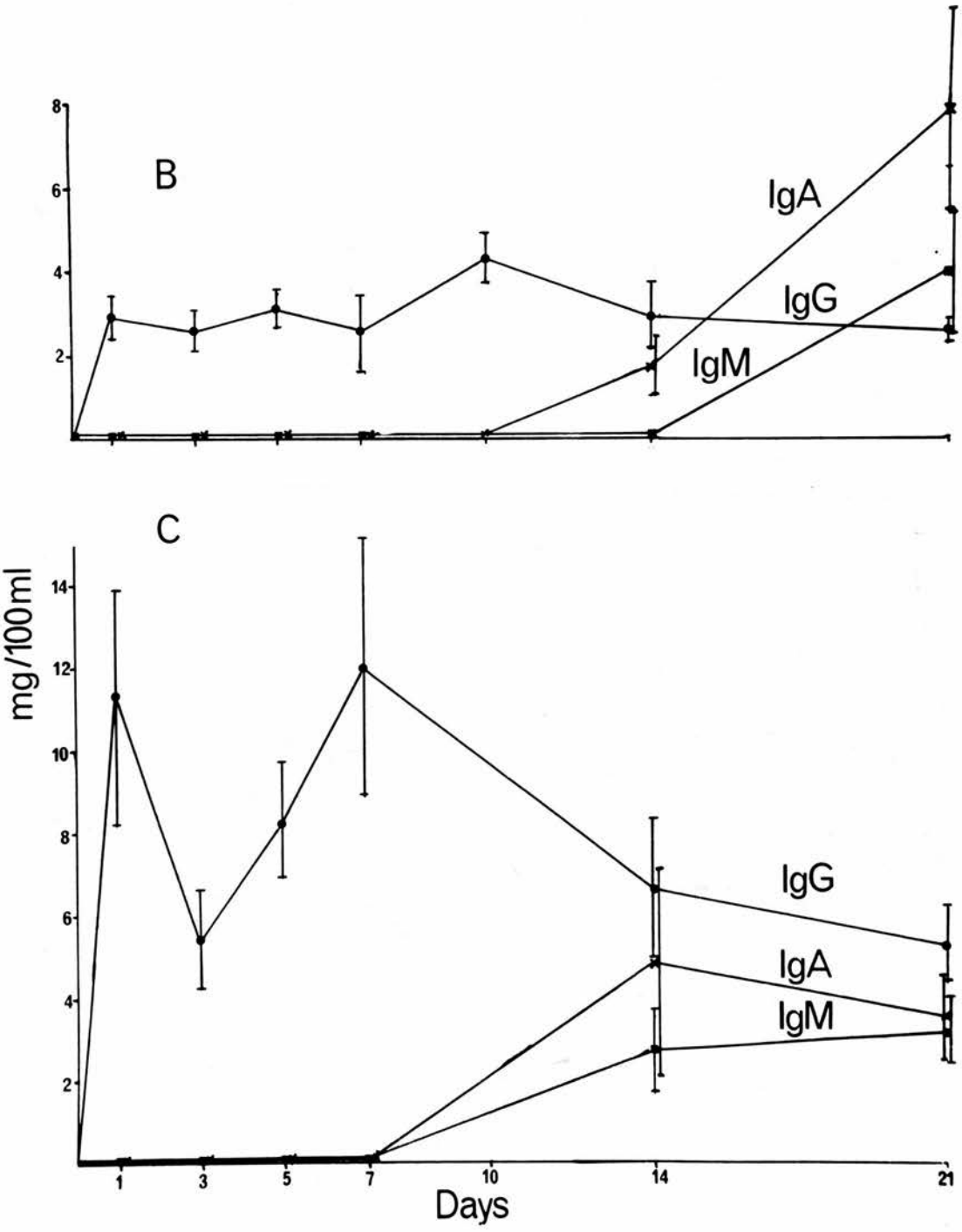

B
IgA
IgG
IgM
8
6
4
2
C
mg/100ml
14
12
10
8
6
4
2
IgG
IgA
IgM
1
3
5
7
10
14
21
Days

Fig. 42 Serial changes in the immunoglobulin concentrations of the lachrymal fluid collected from Group C.

 - standard error of the mean.

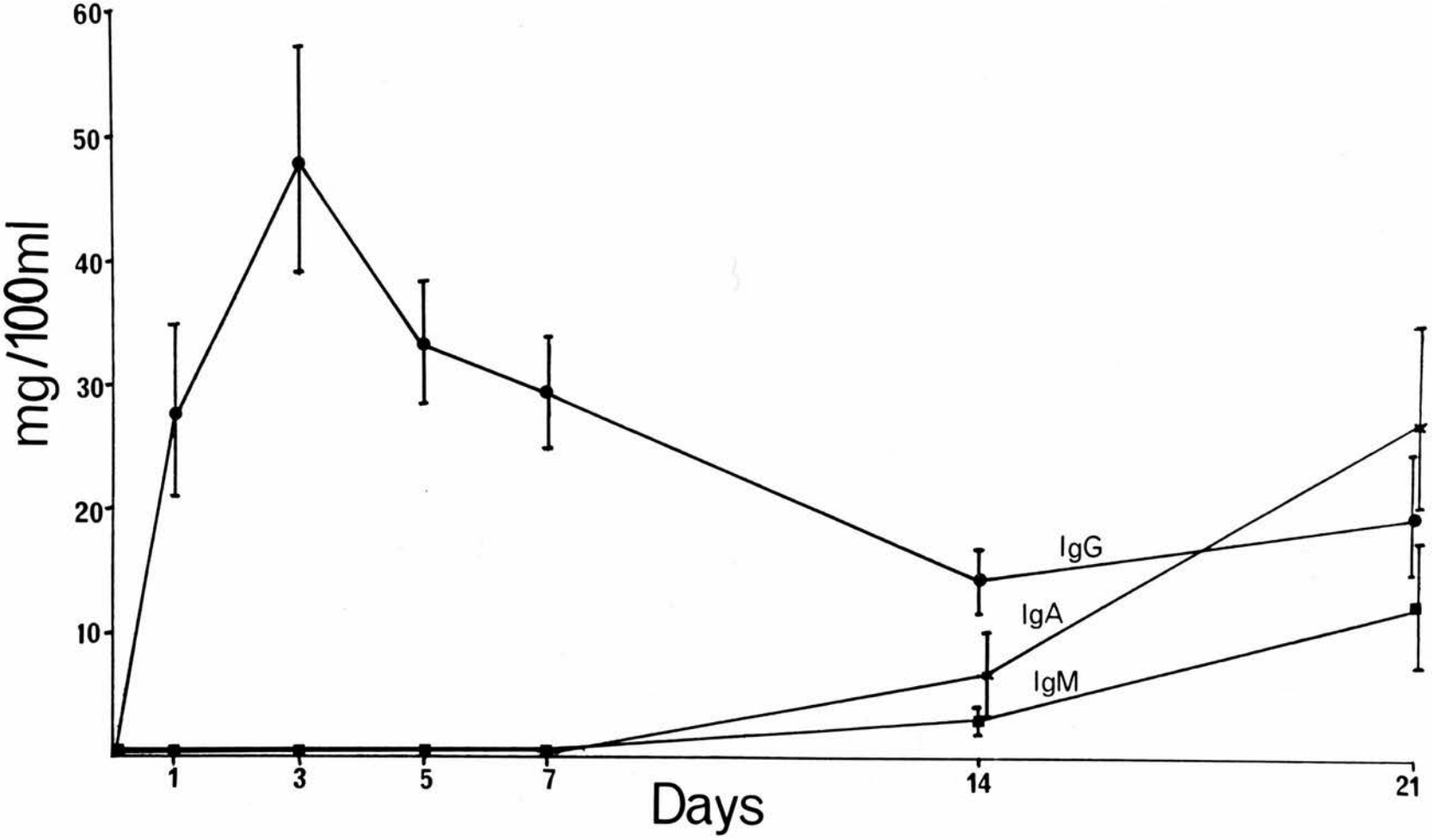

60
50
40
30
20
10
mg/100ml
IgG
IgA
IgM
1
3
5
7
14
21
Days

Table 20

Serum IgG levels and HI titres in ewes and their day-old offspring

Ewe	IgG mg/ml	HI titre	Lamb	IgG mg/ml	HI titre
10399	31.0	10	301	64.0	40
			302	36.5	20
10559	33.0	10	303	48.0	20
			304	94.0	40
10689	34.0	20	305	45.0	40
			306	8.8	10
10500	45.0	20	307	0.9	<10
10328	68.0	10	308	75.0	20
			309	50.0	20
A789	39.0	<10	311	75.0	<10

large amount of colostral IgG but its dam had no detectable serum HI antibody.

Where sufficient sample was available, precolostral and day 1 nasal secretions from lambs in Group A were tested for HI activity. Titres of up to 8 were detected in all precolostral samples tested, but no rise was noted on day 1 despite the finding that some lambs had serum titres of 40. Virus neutralising activity was not detected in any precolostral or day 1 samples.

Fractionation of Nasal Secretions. Fractionation by gel filtration of a pool of nasal secretion samples collected on days 1 to 14 from lambs in Group A showed that HI activity was confined to the protein peak eluted at the void volume of the column (Fig. 43). No IgA or IgM was detected in these fractions. However, virus neutralising activity was also demonstrated in this region, and in fractions eluted after the IgG peak. Fractions containing IgG had no detectable antibody activity. Pooled concentrated nasal washings from lambs less than one week old were filtered through the same column. IgG eluted over the same volume as in Fig. 43 was the only immunoglobulin detected.

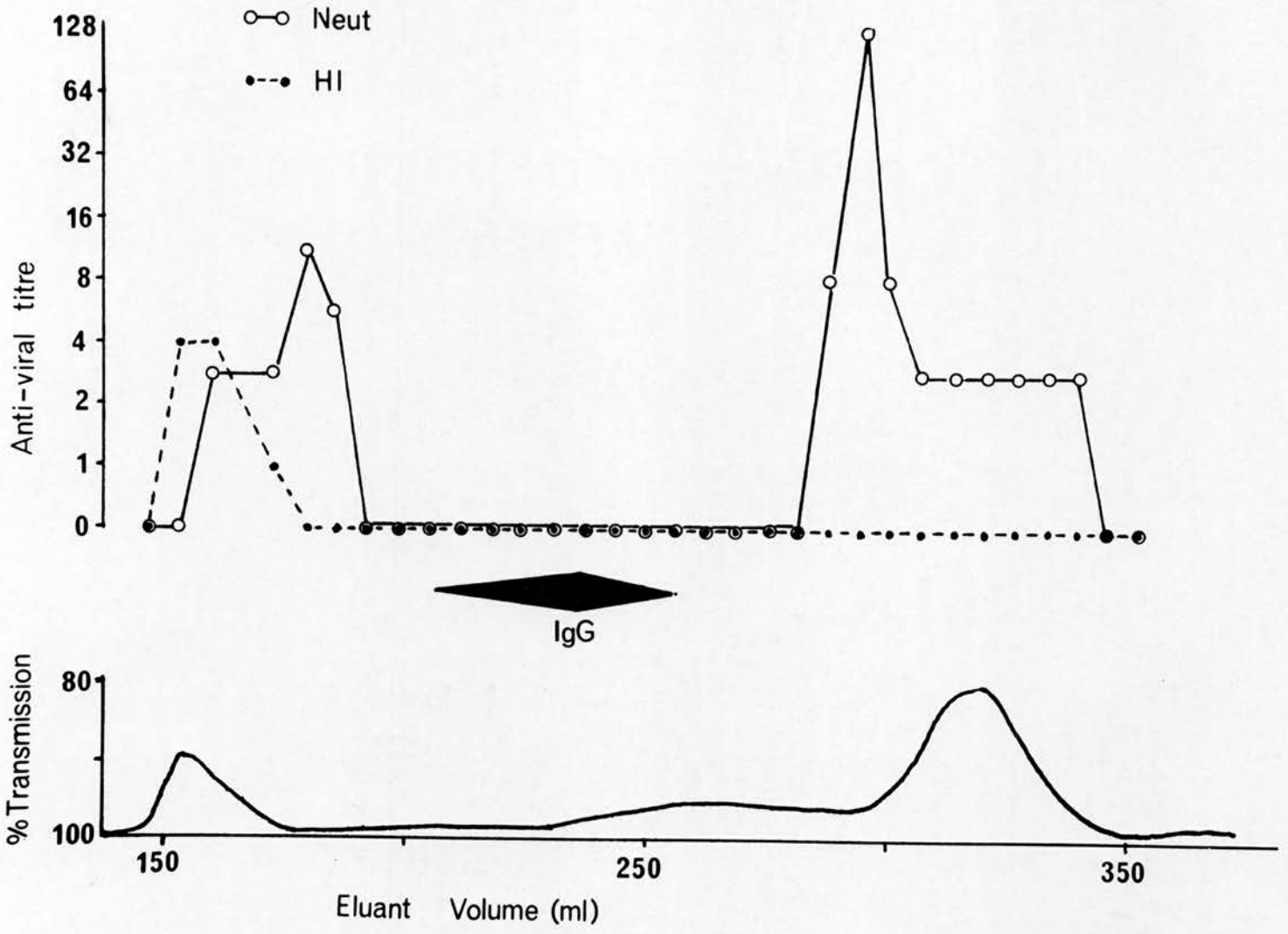
Discussion

Precolostral serum concentrations of immunoglobulin varied widely and, as shown in calves (Penhale et al, 1973), it is clear that lambs are not entirely agammaglobulinaemic at birth. Indeed it has been shown that the ovine foetus is immunologically competent as early as the 66th day of gestation (Silverstein et al, 1963) and it seems possible that the relatively high levels of IgG and IgM observed in certain lambs prior to suckling were the result of an antigenic stimulus in utero, possibly due to an infectious agent. High serum immunoglobulin concentrations have been found in lambs born from ewes

Fig. 43 Fractionation of nasal secretions by filtration through Biogel A 1.5 m.

Sample: 3.7 ml of pooled nasal secretions obtained from the lambs in Group A after they had sucked.

Column: 70 x 2.5 cm. Fraction size: 12 ml.


Neut
HI
128
64
32
16
8
4
2
1
0
Anti-viral titre
IgG
80
100
% Transmission
150
250
350
Eluant Volume (ml)

infected with Border Disease agent (C. Gardiner, Personal Communication). IgM was the most consistently detected and usually the most abundant immunoglobulin in the precolostral serum samples as noted by Cole and Morris (1971). However the level of serum IgG was so high in the first sample collected from one lamb (No. 320) that the efficiency of surveillance may be questioned and it could be suggested that this lamb had sucked. There is no evidence that this was the case and this anomaly remains unexplained.

The great variation in serum immunoglobulin concentrations in individual lambs after sucking is a reflection on the amount and quality of colostrum ingested as well as the time at which sucking first occurred. No relationship was evident between serum immunoglobulin levels in day-old lambs and the concentrations in the colostrum of their dams. Similar findings have been reported by Harker (Personal Communication) and in cattle by Penhale et al (1973).

Six of the 34 lambs (18%) were considered hypogammaglobulinaemic. A similar proportion of lambs were reported to be hypogammaglobulinaemic in a survey carried out in 72 lambs from Scottish Blackface ewes on a commercial hill farm, (Reid, 1972), and Harker (1974) found 19% of lowland lambs hypogammaglobulinaemic. The dam of one hypogammaglobulinaemic lamb in the present study had an indurated mammary gland, but no obvious explanation could be found for the hypogammaglobulinaemia in the other lambs.

Considerable individual variation was found in the serum immunoglobulin levels of twin lambs as reflected in the serum titres to PI_3. Faye et al (1967) previously reported variation in the PI_3 HI titres of sibling lambs. This variation in twin lambs may be due to unequal vigour at birth with the first born having a better chance to ingest

colostrum. In some cases the variation in IgG and IgA levels in the sera of twin lambs was not paralleled by the variation in IgM levels. This may have been due to the time after birth when the colostrum was first ingested, because it has been reported in calves that absorption of the different immunoglobulins from the gut stops at different times: estimated as 27 hours for IgG, 22 hours for IgA and 16 hours for IgM (Penhale et al, 1973). Thus, if a similar situation exists in lambs, a twin lamb which for example received a small amount of colostrum before 16 hours would be expected to have relatively lower serum IgA and IgG levels but relatively higher IgM levels than its sibling which absorbed a larger amount of colostrum after 16 hours.

The half life of 13.5 days for maternal IgG is very close to the value of 13.7 days recently obtained for the decline in lambs' maternal antibody titres to louping ill virus (Brotherston and Boyce, Personal Communication) and Nansen and Aalund (1972) recorded half lives of about 10 days for radiolabelled IgG_1 and IgG_2 in sheep. In the calf, colostral IgG appears to decline more slowly than in the lamb, but the half lives for IgM and IgA are very similar to those estimated here (Logan et al, 1973; Porter, 1972; Husband et al, 1972).

The finding that IgG was present in the nasal secretions or nasal washings after suckling shows that colostral IgG passes into the nasal secretions. Since this occurred in lambs fed immunoglobulin-free artificial milk, the source of the nasal fluid IgG could not be from inhalation of natural milk IgG during sucking. IgG was also found in the lachrymal secretions of newborn lambs after suckling as previously reported (Sullivan et al, 1969). Neither IgA nor IgM was detected in these fluids before the lambs were one week old. Therefore, it seems that the nasal and conjuctival mucous membranes, are unlike those of

the gut which are permeable to all immunoglobulins in the first few hours after birth. Gel filtration analysis showed that the IgG in nasal secretions or washings was eluted in a similar position to serum IgG, indicating that the intact immunoglobulin molecule passes into the nasal mucus. In contrast the urine of newborn suckled calves contains only Fc and Fab fragments of IgG (Kickhofen et al, 1971).

The mechanisms whereby colostral IgG passes into lachrymal and nasal secretions are unknown. However, it is well known that IgG_1 is selectively transferred from serum into bovine or ovine colostrum (MacKenzie and Lascelles, 1968). More recently selective transfer of IgG_1 has been noted in ovine saliva and milk and in bovine lachrymal and other mucous secretions (Watson and Lascelles, 1973 a and b; Pedersen, 1973; Curtain et al, 1971). This mechanism could account for the presence of maternal IgG_1 in the nasal secretions of newborn suckled lambs, since the predominant colostral immunoglobulin is IgG_1. The gradual appearance of IgA and IgM in the nasal and lachrymal secretions is presumably due to local active production of these immunoglobulins.

The concentration of the immunoglobulins in nasal washings was much lower than the concentration in nasal secretions collected by tampons. The use of tampons is a more efficient method of collecting nasal mucus, as was further shown when IgM and IgA were detected earlier in Group A nasal secretions. The possibility that the increased concentration of immunoglobulin in the nasal secretions was due to the tampons causing trauma to the mucosa with consequent transudation of serum immunoglobulins can be excluded since IgA and IgM, which were present in serum, were not found in 1 or 3 day old nasal secretions.

Mean IgG levels in the nasal secretions of day-old lambs were about 1/50 of the mean serum levels. Since the highest serum HI titre in the lambs in this group was 40, this may account for the fact that no rise in titre was recorded in the nasal secretions after sucking. Fractionation confirmed that PI_3 antibody could not be detected in pooled nasal secretions. However HI activity was found in the exclusion peak from gel filtration. This activity was presumably caused by the same inhibitor present in precolostral samples and previously detected in nasal secretions from specific pathogen free and conventionally reared lambs (Section III). Since IgM was not detected in these fractions in this experiment, the activity was unlikely to have been due to IgM antibody. The first peak of neutralising activity found in the nasal secretion fractions was assumed to be caused by the same inhibitor. However the cause of the neutralising activity eluted after IgG is not known.

McKercher and collaborators (1972) found neutralising activity to PI_3 in the nasal secretions of 3 month old calves. This activity was assumed to be due to maternal antibody reaching the nasal secretions. The situation was not clear however, since neutralising activity was also found in the nasal secretions of some colostrum deprived calves. The nasal secretions of calves have also been examined for maternal antibodies to rinderpest virus (Provost, 1970) and, while no antibody was found in the nasal secretions of older calves, 2 one month old calves with serum titres greater than 100 did have nasal secretion neutralising antibody. Nasal antibody was not detected in a third 1 month old calf without serum antibody. Bernhardt and Bengelsdorff (1973) found HI activity to PI_3 in the nasal secretions of two 8 day old colostrum fed calves. They attributed this activity to maternal antibody which had passed into the secretions, but presuckling samples were

not tested and the activity was not shown to be due to immunoglobulin. Findings in this experiment, together with a report describing HI activity in colostrum deprived calves nasal secretions(Morein 1972), suggest that Bernhardt and Bengelsdorff may have been measuring non-specific inhibitors.

S E C T I O N V

GENERAL DISCUSSION

Ovine IgA, IgM and IgG and antisera specific for these immunoglobulins were prepared in the first part of the experimental work described in this thesis (Section II, part 1). These reagents were used to determine the levels of each immunoglobulin in the respiratory secretions and certain other body fluids of adult sheep. The results showed that IgA was the major immunoglobulin in saliva and in the respiratory and lachrymal secretions while IgG was the predominant immunoglobulin in colostrum, milk, bile and serum. The immunoglobulin concentrations found in serum, saliva and mammary secretions approximated values reported by others for the sheep (Pahud and Mach, 1970; Watson and Lascelles, 1973 a and b). No reports for sheep were available to compare the results obtained for the other fluids examined, but the concentrations of IgA, IgG and IgM found in nasal secretions, tears and bile were approximately equivalent to those estimated for the bovine (Mach and Pahud, 1971; Duncan et al, 1972).

Lung fluid from sheep clinically affected with pulmonary adenomatosis was a useful source for preparing IgA since it contained high concentrations of IgA with a high IgA:IgG ratio. Serum from such sheep contained the normal high IgG:IgA ratio, showing that the lung fluid was not a simple transudate from plasma. IgA antibodies to Mycoplasma ovipneumonia were present in the lung fluid, however the significance, if any, of these antibodies and the function of this large amount of IgA remain to be elucidated.

Thus the results from Section II of this thesis established that an IgA antibody system existed in the ovine respiratory tract. Mean IgA concentrations were higher in the respiratory secretions than serum, suggesting that this immunoglobulin was either locally

produced in the respiratory tract or selectively derived from serum. However fluorescent studies on other ovine mucous surfaces (Lee and Lascelles, 1970; Curtain and Anderson, 1971), and immunochemical measurements on sheep milk and saliva (Watson and Lascelles, 1973 a and b) suggest local production of IgA and there is no reason to suspect a different situation in the respiratory tract.

Since IgA antibodies to mycoplasma had been found in lung fluid, attempts were made to detect locally produced antibody in the respiratory secretions of 4 lambs IN inoculated with live PI_3 virus. The results showed that neutralising antibody was present in the nasal secretions of 3 lambs 5 days after instillation of the virus, whereas in the remaining lamb significant titres were not recorded before day 18. Nasal secretion antibody was usually detected before either neutralising or HI antibody in the serum, but did not persist as long as serum antibody, and could not be detected consistently by 3 to 4 weeks post inoculation. Marked day to day variations occurred in the concentrations of IgA, IgG and IgM in the nasal secretions collected from both infected and control lambs. Thus it was not possible to associate the appearance of nasal antibody with an increase in immunoglobulin levels. However analysis of nasal and tracheo-bronchial secretion pools by gel filtration revealed that the bulk of specific anti-viral activity in the respiratory secretions was associated with IgA. Thus it was concluded from the experiment that sheep can manufacture locally produced IgA antibodies after infection with a respiratory virus.

Detection of specific IgA antibodies was complicated by a non-antibody inhibitor of PI_3 also present in these secretions This inhibitor was not due to a factor present in the tampons used

to collect the secretions and was later found in newborn and SPF lamb nasal secretions, devoid of detectable immunoglobulin (Section III, part 2, Section IV, part 2). It inhibited the HA of PI_3, PI_2, PI_1 and NDV and was only partially sensitive to treatment with RDE. This substance mainly inhibited HA but when concentrated also neutralised PI_3. Similarly Gates et al (1970) recorded non-specific neutralisation of PI_3 with concentrated nasal washings from calves. However pre-inoculation nasal secretions and secretions from control lambs did not neutralise the virus and so this test was used to detect antibody in unfractionated secretions.

Sheep saliva contains a well characterised large molecular weight glycoprotein, which is excluded from G200 Sephadex and which inhibits the HA of influenza virus (Bhargava et al, 1966; Gottschalk et al, 1972). The inhibitor found here in ovine nasal secretions was estimated by gel filtration to have a molecular weight of about 10^6 and may be a similar substance.

It is of interest to compare the findings in this sheep experiment with reports of PI_3 specific IgA antibodies in bovine nasal secretions. Fractionation of bovine nasal washings using G200 Sephadex has revealed a major exclusion peak which contains IgA (Morein, 1970; Duncan, et al, 1972). Morein (1970) found PI_3 HI activity in nasal washings from naturally infected cattle and, after fractionation of these washings using Sephadex G200, he concluded that the nasal wash HI activity was due to specific IgA antibody. Morein, (1970) also reported that the PI_3 HI titre of the nasal washings was proportional to the protein content of the sample, a finding which implied that any increase in the HI titre would be paralleled by higher levels of IgA. Gel filtration of nasal

secretions of lambs in this study was carried out on Biogel A 1.5 m which gives greater separation of IgM, IgA and IgG than Sephadex G200 (Section III, Materials and Methods). Results indicated that the PI_3 HI activity in the respiratory tract secretions of lambs was mainly due to a non-immunoglobulin protein which was eluted before IgA and which was the major protein in the secretions, as estimated by absorption at 254 nm (Sections III and IV). Furthermore after infection of these conventionally reared lambs and the SPF lambs (Section III, part 2), no rise in nasal secretion IgA levels were found.

These results suggest that certain reported findings of IgA antibodies with PI_3 HI activity in bovine nasal washings may have been due, in part, to non-immunoglobulin inhibitors (Morein, 1970; Mesnik et al, 1971). Indeed, Morein (1972) later reported that the nasal secretions of day-old colostrum deprived calves inhibited the HA of NDV, PI_3 and PI_1 viruses. He detected IgA in these nasal secretions and implied that the HI activity might be due to this 'early IgA' functioning as a non-specific inhibitor as suggested by Alford et al (1967). No IgA was detected in any of the newborn lambs' nasal secretions examined in this thesis (Section IV, part 2). It is not clear whether Morein ruled out the possibility that his anti-IgA serum reacted with free secretory component, which may have been present in the nasal secretions of the newborn calves. Free secretory component, without detectable IgA, has been found in the saliva of newborn calves (Mach and Pahud, 1971).

After parenteral or IN vaccination of calves Morein (1972) found increased nasal secretion HI titres to PI_3 and PI_1 viruses but not to NDV. He thought this might reflect a partial antigenic

relationship between the parainfluenza viruses or a broader specificity of IgA as suggested by Waldman et al (1970a). In contrast, no PI_1, PI_2 or NDV HI activity ascribed to IgA activity was found in the nasal secretions of the lambs which were inoculated with PI_3 in the present study (Section III, part 1).

However more recently, IgA has been prepared from calf nasal secretions by ion exchange and gel filtration chromatography, and only IgA molecules from calves which had been infected with PI_3 were shown to attach to PI_3 virions as visualised by electron microscopy (Morein et al, 1973). In accord with findings in calves, no PI_3 specific IgG antibody was found in ovine nasal secretions after IN infection with virus (Morein, 1972).

It was considered that the IgA antibody system in the respiratory secretions might be an important defence mechanism in preventing respiratory infections of sheep. Attempts were therefore made to define the role of nasal secretion antibody to PI_3 with vaccination-challenge experiments in SPF lambs. For convenience of sampling, nasal secretions were chosen as a model respiratory secretion. SPF lambs were used so that previous or intercurrent infections with PI_3 could be excluded. It was thought that changes in serum and nasal secretion immunoglobulin levels would be more evident in SPF rather than colostrum deprived conventionally reared lambs which would be exposed to many more antigens. In addition young SPF lambs were considered likely to be clinically susceptible to challenge with PI_3, since respiratory tract lesions had previously been produced in new-born lambs (Hore and Stevenson, 1967). Challenge virus was administered by aerosol because this was considered the likely route of natural infection. Furthermore clinical disease had been

produced in calves experimentally challenged with aerosols of PI_3 (Frank and Marshall, 1971).

However a complete absence of clinical disease was found when these lambs were exposed to PI_3 either IN, IM or by aerosol (Section III, part 2). Thus virus excretion from the nose was used as an index of challenge immunity. Previously this method had been employed in calves (Gates et al, 1970; McKercher et al, 1972) and Gilmour et al, (1968) showed that the technique could be applied to PI_3 in sheep. Using this criterion for measuring protection, a preliminary dose of live virus given either IN or IM protected the lambs against subsequent aerosol challenge. Live virus administered by either route stimulated similar serum antibody titres, but nasal secretion antibody was only observed after IN inoculation. However, although no virus was recovered after challenge from lambs vaccinated by either method, no post challenge changes in nasal antibody were found in the IN group indicating that they were resistant, whereas a marked increase in titre occurred in the IM vaccinated group and in the other vaccinated groups which shed virus after challenge. Possibly the group IM vaccinated with live virus had a low grade post-challenge infection, which stimulated a nasal antibody response but was undetected by the virus isolation technique.

McKercher et al, (1972) reported equivalent post-challenge nasal antibody changes in colostrum deprived calves vaccinated IM or IN with a living attenuated PI_3 vaccine. Others have demonstrated in calves that nasal secretion antibody was better stimulated by IN vaccination of live virus (Marshall and Frank, 1971; Morein, 1972). and have shown that this route is more protective than IM inoculation (Gates et al, 1970; Gutekunst et al, 1969; Frank and Marshall, 1971).

No protection and no antibody was observed in lambs vaccinated either once or on 2 occasions with inactivated virus IN. Poor antibody responses have also been reported in calves vaccinated with inactivated PI_3 by the respiratory route (Marshall and Frank, 1971; Morein et al, 1973).

IM vaccination with inactivated virus alone elicited low serum titres only and was not protective. However emulsification with FCA rendered the inactivated virus highly immunogenic, stimulating high serum and nasal antibody titres, which increased to very high levels after subsequent vaccination, either IM with FCA, or IN without adjuvant (Section III, experiment 2). Nasal secretion antibody titres were greater, albeit temporarily, when the second vaccination was given IN. However both vaccination methods conferred protection against infection since virus was not recovered from either group of lambs after challenge.

There was some evidence that the presence of high circulating immunoglobulin levels prior to parenteral vaccination might reduce the resulting nasal secretion antibody titre (Section III, experiment 3). However Gilmour et al (1968) found that 2 subcutaneous injections of inactivated PI_3 in incomplete Freund's adjuvant stimulated serum antibody in young adult sheep and conferred protection against challenge infection. However nasal secretion antibody was not assayed in that investigation.

After the SPF lambs were exposed by aerosol to PI_3, increased concentrations of IgM were associated with the primary serum antibody response and increased concentrations of IgG with the secondary response. However no changes in nasal secretion immunoglobulin concentrations were associated with nasal antibody after

infection with the virus, although all 3 immunoglobulins were present in the secretions. Morein (1972) found IgA, IgG and IgM in the nasal secretions of young colostrum deprived calves which had been infected with PI_3, but since he did not examine control animals, it was not clear whether these immunoglobulins were produced in response to the infection.

Some evidence was found for an anamnestic nasal antibody response in the lambs, in the first experiment in Section III. Thus, with the exception of the IN vaccinated lambs which were considered solidly immune, nasal antibody appeared more quickly in all vaccinated groups after challenge compared with the unvaccinated control lambs. Unfortunately it was not possible to determine which immunoglobulins were involved in this response. Evidence for the existence of an anamnestic IgA antibody response in man and laboratory animals is conflicting (Ogra and Karzon, 1969 a; Buscho et al, 1972; Gerbrandy and van Dura, 1972), but data clearly demonstrating an anamnestic nasal antibody response to PI_3 has been presented for the bovine, although it was not shown whether this response was IgA mediated (Marshall and Frank, 1971; Woods et al, 1973).

Following vaccination of the lambs with inactivated virus in FCA, high levels of antibody and IgG were observed in the nasal secretions and serum (Section III, experiment 2). Fractionation by gel filtration and immunoelectrophoresis confirmed that the bulk of serum antibody was associated with IgG (IgG_1 and IgG_2), whereas the nasal secretion antibody was IgG_1 only. This was an interesting contrast to the earlier finding that IgA antibodies were produced following IN instillation of live virus (Section III, part 1). It is suggested that the effect of FCA was to stimulate high levels of

circulating IgG_1 and IgG_2 antibodies and that the IgG_1 subclass was selectively transferred into the nasal secretions. This proposal is likely in view of the recent evidence accumulating from immunochemical and radiolabelling studies with IgG_1 and IgG_2, showing that IgG_1 is selectively transferred into a number of sheep and cattle body fluids including respiratory, lachrymal, lacteal, cervicovaginal and salivary gland secretions (Curtain et al, 1971; Duncan et al, 1972; Pederson, 1973; Watson and Lascelles, 1973 a and b). These experiments were not conducted with regard to micro-organisms and specific antibody titres were not measured. However 2 recently reported studies with cattle show equivalent results to the findings made in the present investigation with the lambs and PI_3. Garland (1974) reported that antibodies in the nasal secretions of cattle recovered from foot and mouth disease were of the IgA type, but, after subcutaneous immunisation with inactivated foot and mouth disease vaccine, the secretion antibodies were largely IgG. Similarly Corbeil et al (1974) found IgA antibodies in the cervicovaginal mucus of heifers locally infected with Vibrio foetus, whereas cattle parenterally immunised with killed organisms in FCA had IgG antibodies in the vaginal mucus. In contrast Morein (1972) associated the HI activity in the nasal secretions of parenterally immunised calves with IgA antibody. For reasons already mentioned, this apparent discrepancy of results might be explained if some of the HI activity Morein was measuring was due to non-antibody inhibitors. Others have detected low levels of neutralising antibody in the nasal secretions of older calves, parenterally immunised with PI_3, but the immunoglobulin type or source of this antibody was not determined (Marshall and Frank, 1971;

McKercher et al, 1972).

No delayed-type skin hypersensitivity reaction was seen in lambs experimentally infected with PI_3, but the results were not conclusive (Section III, part 1). Positive intradermal tests have been demonstrated in cattle naturally infected with PI_3 (Morein and Moreno-Lopez, 1973) and systemic delayed hypersensitivity has been reported in guinea pigs IN infected with this virus (Wetherbee, 1973). Cell mediated immunity to influenza virus has been detected in alveolar lymphocytes from humans and guinea pigs (Jurgensen et al, 1973; Waldman et al, 1972; Gadol et al, 1974), and it seems likely that this type of immunity may also play a role in preventing infection of the respiratory tract.

In the vaccination-challenge experiments with the SPF lambs high antibody titres correlated with protection against infection with PI_3, but it was not clear whether antibody was in fact responsible for the protection conferred, because cellular immune mechanisms were not monitored. This question was resolved by challenging very young colostrum fed lambs, which were unlikely to have been infected with PI_3 and so would lack active immunity to the virus. PI_3 was recovered on significantly ($p = < 0.05$) fewer occasions from 3 lambs with high levels of maternal antibody, compared with 5 lambs with low levels of antibody. Thus, although the number of animals involved was small, this experiment indicated that antibody alone could prevent lambs becoming infected with this virus.

Prechallenge samples of nasal secretions from these lambs contained IgG and samples from 2 lambs also contained significant titres of neutralising antibody. It seemed likely that this immunoglobulin was of maternal origin and might constitute an important

defence mechanism in newborn lambs. A more detailed investigation was therefore carried out involving 34 lambs - the offspring of 19 ewes (Section IV, part 2). The results confirmed that colostral IgG did pass into the nasal secretions of these lambs. Precolostral samples were devoid of immunoglobulin, whereas after sucking IgG only was detected in nasal secretions, nasal washings and tears. Maternal IgG was also present in these fluids if lambs were removed from their dams after 24 hours and subsequently reared on immunoglobulin-free milk substitute. This proved that the IgG present in these fluids was not derived from milk inhaled during suckling. Since IgG was present in the nasal washings as well as nasal secretions, possible trauma, resulting from the use of tampons, was ruled out as the cause of transudation.

IgG concentrations in the nasal secretions were about 1/50 of serum concentrations and declined as the lambs grew older in contrast to the situation in the colostrum deprived SPF lambs (Section III, part 2). IgA and IgM were first detected in the secretions about 10 days after birth, presumably as a result of local antibody synthesis. Sullivan et al (1969) reported transfer of colostral IgG into the tears of newborn lambs, but these authors did not have specific anti-IgA and anti-IgM sera and could not determine whether the newborn conjunctiva was selectively permeable to IgG or was permeable to all immunoglobulins, like the gut. The finding of colostral IgG (which is mainly IgG_1) in the nasal secretions supported the earlier suggestion that the IgG_1 antibody found in the adjuvant vaccinated lambs' nasal secretions was selectively derived from plasma.

Serum immunoglobulin levels were also monitored in the lambs sampled in this experiment. Pre-suckling samples confirmed

other findings that lambs are not completely agammaglobulinaemic at birth (Gardiner, Personal Communication). In most lambs large increases in serum immunoglobulin concentrations were recorded after birth, but almost 20 percent remained hypogammaglobulinaemic - a proportion similar to that found in two other recently reported surveys (Reid, 1972; Harker, 1974). Hypogammaglobulinaemia was presumably caused by poor ingestion of colostrum, but, with one exception, the reasons for this were obscure. This relatively high incidence of hypogammaglobulinaemia is likely to be a major cause of loss in young lambs as a result of infectious disease. Increased susceptibility to infection was well illustrated in Section IV, part 1 where one lamb ~~ingested~~ possessed about 1/5 the amount of ~~colostrum~~ colostral antibody received by its twin rendering it susceptible to challenge infection with PI_3 whereas its sibling remained immune.

Colostral immunoglobulins reached peak levels in the lambs' sera approximately 24 hours after first suckling. IgA and IgM declined relatively quickly with mean half lives of 1.8 and 4.1 days respectively. Comparable results have been recorded for the rate of decline of bovine colostral IgA and IgM in calves' sera (Logan et al, 1973; Porter, 1972; Husband et al, 1972). However the results indicated that colostral IgG declines more rapidly in the lamb than in the calf, since a mean half life value of 13.7 days was obtained, compared with a figure of 21 days reported for calves (Logan et al, 1973).

The ewes and lambs sampled in this experiment had low serum titres to PI_3. Therefore, since peak maternal IgG levels in the nasal secretions were approximately 1/50 of the serum concentration, maternal antibody to PI_3 was not detected in the nasal secretions.

Presumably antibody was present at undetectable levels in the nasal secretions, especially since antibody was detected in secretion samples collected from 2 lambs with high serum titres in the previous experiment, (Section IV, part 1). Low levels of maternal antibody specific for other respiratory micro-organisms were probably also present in the nasal secretions of the newborn lambs. These may play a valuable role in providing transient protection against respiratory tract infections, before active production of local IgA and IgM begins at about 2 weeks of age (Sections III and IV). The same situation might apply to other mucous surfaces including the conjunctiva.

The protective role of nasal secretion antibody per se is difficult to evaluate from the results in this thesis. All lambs which had nasal antibody and were immune to PI_3 also had serum antibody. However IN vaccination with live virus stimulated higher nasal secretion titres than live virus administered IM and the IN vaccinated lambs were considered more resistant to challenge, despite similar pre-challenge serum titres in both groups. On the other hand some lambs with serum antibody and without detectable nasal antibody were also immune to challenge infection (Section IV).

Protection against parainfluenza virus infections in man and cattle has been found to correlate more closely with antibody titres in the nasal secretions (Smith et al, 1966; Frank and Marshall, 1971). Therefore it is reasonable to assume that antibody in ovine nasal secretions plays some role in preventing infection with this virus and possibly with other micro-organisms which replicate in the respiratory tract. It seems that antibody in

lambs' nasal secretions can be stimulated in 2 ways: either, 1) by IN administration of live virus or, 2) by parenteral immunisation with inactivated virus in adjuvant. The first method stimulates IgA antibody which is probably locally produced. The second method results in IgG_1 antibody reaching the nasal secretions, probably by selective transport from serum. The finding of colostral IgG (which is mainly IgG_1) in newborn suckled lambs' nasal secretions supports this last suggestion. Other recent evidence is available which suggests that cattle have these 2 mucous antibody systems (Garland, 1974; Corbeil et al, 1974).

IgA and IgG from bovine cervico-vaginal mucus have been shown in vitro to have different functional properties with respect to Vibrio foetus organisms (Corbeil et al, 1974), but it is not known if IgA and IgG_1 anti-viral antibodies have similar in vivo properties in the nasal secretions of lambs. In terms of highest neutralising antibody titres stimulated, IM vaccine with FCA followed by an IN 'booster' was the best vaccine combination. However it remains to be seen if this method will be of value in conventionally reared lambs, particularly since high levels of serum IgG seemed to inhibit the amount of antibody reaching the nasal secretions (Section III, experiment 3). If a practical vaccine of this type were to be developed then obviously an adjuvant less noxious than complete Freunds' would have to be found. Trials would have to be run to ensure that the anaphylaxis found in this study would not occur following natural infection with PI_3.

An alternative vaccination method would be to use live attenuated virus administered IN. Maternal antibody is less likely to interfere with this type of vaccination, particularly if vaccination is delayed

until the lambs are over one month old. By this time maternal antibody should have disappeared from the nasal secretions and the respiratory IgA system should be immunocompetent. However more work is necessary to elucidate the duration of the IgA nasal antibody response and more direct evidence is required to prove that sheep are capable of an anamnestic respiratory IgA response.

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List of Abbreviations Used.

DEAE	-	diethylaminoethyl
FCA	-	Freund's complete adjuvant
HA	-	haemagglutination, haemagglutinin
HI	-	haemagglutination inhibition
IEP	-	immunoelectrophoresis
IM	-	intramuscular (ly)
IN	-	intranasal (ly)
NDV	-	Newcastle disease virus
PBS	-	phosphate buffered saline
PI_1	-	parainfluenza one
PI_2	-	parainfluenza two
PI_3	-	parainfluenza three
RDE	-	receptor destroying enzyme
SAS	-	saturated ammonium sulphate
SPF	-	specific pathogen free
$TCID_{50}$	-	tissue culture infective dose fifty

ACKNOWLEDGMENTS

I wish to thank Dr. A.E. Stuart my supervisor for his kindly interest, advice and encouragement, given to me during the course of this work.

I am extremely grateful to Dr. W.B. Martin, Dr. P.W. Wells, Mr. A. McL. Dawson and Mr. C. Burrels without whose continuous help and constructive criticism this project would not have been possible. I am also indebted to many other colleagues at Moredun for their advice, help and ideas.

Finally I wish to thank Dr. J. Stamp for the facilities and opportunities provided at Moredun Institute and the Animal Diseases Research Association for their financial support.

Appendix 1.

Preparation of the buffers used in DEAE cellulose chromatography

0.2M stock solutions of Na H_2PO_4. H_2O and NaH PO_4 were made up.

0.2M phosphate buffer (PO_4) was then prepared by adding the dihydrogen salt to the disodium salt until the pH fell to 7.6.

Thus 0.01M PO_4 was obtained by diluting 0.2M PO_4 1:20 with distilled water.

A stock solution of 0.2M NaCl was made up and thus 200 ml of e.g 0.01M PO_4, 0.01M NaCl (buffer step 1) was prepared by adding 10 ml of 0.2M (PO_4) to 10 ml 0.2M NaCl and making up to 200 ml with distilled water.

Appendix 2

Publications arising from work described in this thesis

1) Smith, W.D. The Nasal Secretion and Serum Antibody Response of Lambs following Vaccination and Aerosol Challenge with Parainfluenza 3 virus. Research in Veterinary Science, in press.

2) Smith, W.D., Dawson, A. McL., Wells, P.W. & Burrells, C. Immunoglobulin Concentrations in Ovine Body Fluids. Research in Veterinary Science, in press.

3) Smith, W.D., Wells, P.W., Dawson, A. McL. & Burrells, C. Immunoglobulins, Antibodies and Inhibitors of Parainfluenza 3 Virus in Respiratory Secretions of Sheep. Research in Veterinary Science, submitted for publication.

4) Smith, W.D., Wells, P.W., Burrells, C. & Dawson, A. McL. Immunoglobulins in the Serum and Nasal Secretions of Lambs following Vaccination and Aerosol Challenge with Parainfluenza 3 Virus. Research in Veterinary Science, submitted for publication.

5) Smith, W.D., Wells, P.W., Burrells, C.& Dawson, A. McL. Maternal Immunoglobulins and Parainfluenza 3 Virus Inhibitors in the Serum and Nasal Secretions of Newborn Lambs. Research in Veterinary Science, submitted for publication.